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14. ABSTRACT

Breast cancer remains a destructive disease despite new therapeutics. It is well accepted that environmental factors can play an important role in determining one's future risk of the disease. We believe that two natural polyphenols, genistein (a component of soy) and resveratrol (a component of grapes and red wine), can suppress mammary carcinogenesis. We and others have clearly shown mammary-protective effects against chemically-induced cancer. This project aimed to elucidate mechanisms through which these polyphenols may exert their effects. We show that genistein and resveratrol modulate the gene and protein expression of several critical players in the mammary gland involved in growth and proliferation. We see changes in MAPK signaling pathway, the PI3K/Akt pathway, apoptotic cascade, beta casein, as well as changes in sex steroid receptor co-activators. We have demonstrated that the estrogen receptors play an important role in the mechanisms of genistein and resveratrol. These data will add to the pre-clinical data necessary to forward further work with these polyphenols towards the prevention of breast cancer.

15. SUBJECT TERMS
Breast Cancer, Chemoprevention, Genistein, Resveratrol

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Introduction:

Breast cancer remains a destructive disease and a leading killer among women in the United States and throughout the world [1]. It has been recognized that genetic alterations (such as BRCA 1 and 2 mutations) account for only 10-15% of breast cancer. Thus, environmental exposures, especially diet, can play a very important role in the causation or prevention of this disease. We believe that the dietary polyphenols genistein, the major phytoestrogen in soy, and resveratrol, a phytoalexin found in red grapes and red wine, can protect a woman against mammary carcinogenesis. We, and others, have shown that dietary exposure to genistein or soy, especially early in life, can protect against chemically-induced carcinogenesis [2-3]. We demonstrated that prepubertal exposure to genistein caused a significant reduction in terminal end buds, the most susceptible structures for mammary carcinogenesis. We and others have also shown a protection against breast cancer in a chemically-induced rat model with exposure in the diet to resveratrol [4-6]. Resveratrol caused a significant reduction in mammary tumor multiplicity and increased tumor latency. The epithelial cells of the terminal end buds show a significant reduction in proliferation and increase in apoptosis, which might help to explain the chemoprotection that we observed [6]. With observations from the tumorigenesis studies, mammary gland maturation, and cell proliferation experiments; we proposed to look for changes at the molecular level that could account for the protection we observe with dietary genistein and resveratrol. We propose to focus on sex steroid and growth factor signaling pathways, and the steroid receptor coactivator family, a possible link between critical sex steroid and growth factor pathways. Also, we want to better understand the role of the estrogen receptors in the chemopreventive mechanisms of genistein and resveratrol. Lastly, we used TaqMan Low Density Arrays to further characterize the mechanisms through which resveratrol and genistein can protect against mammary carcinogenesis. The arrays allow the detection of gene expression levels of 96 genes that are known to play a role in mammary gland development, proliferation, apoptosis, and carcinogenesis simultaneously (Appendix A, Table 2). Changes observed at the genomic level will guide future efforts at the protein and enzymatic levels in the mammary gland. Understanding the *in vivo* mechanisms of these polyphenols will allow them to be used to protect women against breast cancer.

Body:

To discuss the research accomplishments over the three years (Feb. 2005 – Feb. 2008), the Revised Statement of Work will be used with each of three aims being discussed.

Aim 1. (Months 1-12). As a means of understanding how genistein and resveratrol suppress the development of chemically-induced mammary cancer in rats, we proposed to investigate the potential of genistein and resveratrol, alone and in combination, to regulate critical sex steroid receptors, steroid receptor coactivators, and critical growth factors and receptors in the mammary glands of Sprague- Dawley rats.

The fourth abdominal mammary glands were dissected from both 21- and 50-day-old rats treated ± the polyphenols genistein and resveratrol, alone and in combination.

Immunoblot analysis was employed to look at the protein expression of several sex steroid receptors, steroid receptor coactivators, and growth factor receptors. The steroid receptor coactivator GR interacting protein-1 (GRIP-1) was shown to be up-regulated at 21 days postpartum by genistein in the diet (data not shown). This is followed by a decrease in GRIP-1 expression at 50 days postpartum (Figure 1). This fits a model proposed by our lab that genistein causes an increase in mammary gland proliferation and maturation early in the animals, followed later in life by a more mature gland that is less proliferative and thus less susceptible to carcinogenesis [reviewed in 7]. More recently, with genistein treatment, we observed a decrease in the expression of steroid receptor coactivator-1 (SRC-1), another coactivator that enhances steroid receptor signaling, at 50 days postpartum. We did not observe significant differences in the sex steroid receptors such as the estrogen receptors alpha and beta or progesterone receptors at 21 or 50 days postpartum. We did not observe significant regulation of the epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGF-1R), or total and phosphorylated extra-cellular regulating kinase 1&2 (ERK 1&2) at 21 or 50 days postpartum (data not shown).
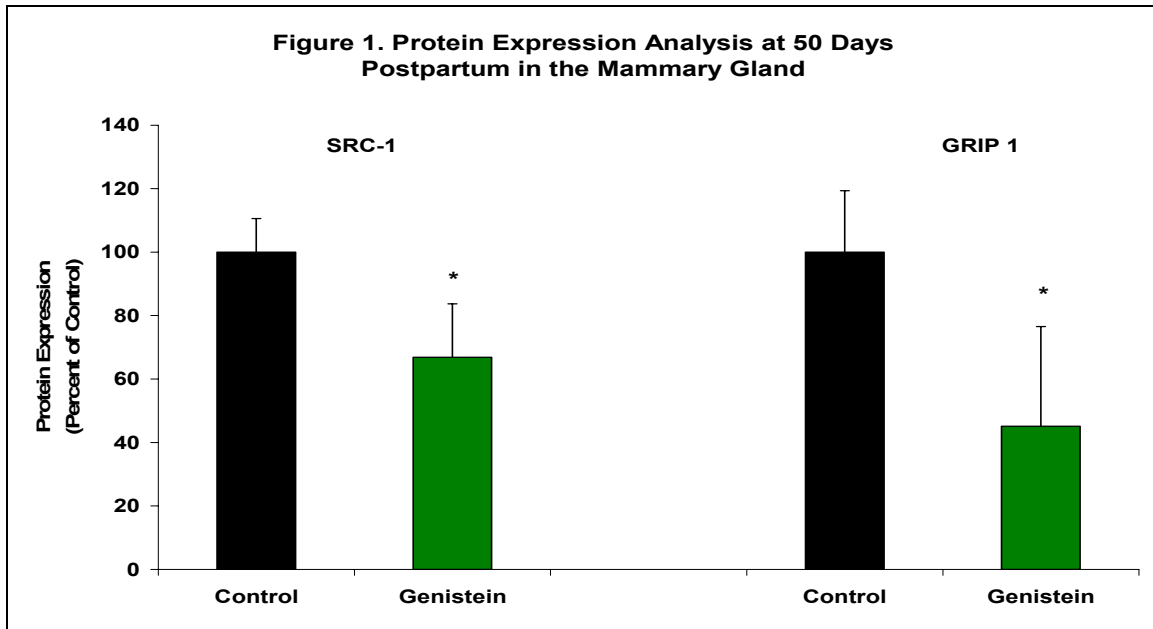
*Figure 1. Rats treated ± genistein (250mg/kg diet) were sacrificed at 50 days postpartum. Mammary glands were dissected and assayed for protein expression by western blot analysis. Results are given as means + SEM, with the Control set to 100. * represents a p value < 0.05*

Resveratrol-treatment resulted in protein modulation which helps to explain the protection that we observed against mammary carcinogenesis. At 50 days postpartum, we observed a significant reduction in the level of GRIP-1 (Figure 2), similar to what we observed with genistein treatment. Furthermore, we detected decreases in protein expression of IGF-1R, Akt, and the phosphorylated (active) form of Akt. All of these have been implicated in mammary cell proliferation and carcinogenesis. A reduction in

these growth factors may help to explain the mammary chemoprevention that we observed in the rat model. Again, we observed no difference in the protein expression of the estrogen receptors or the progesterone receptors at 21 or 50 days postpartum. We saw no changes in the steroid coactivators at 21 days postpartum. At 50 days postpartum, there was a trend toward reduction in the phosphorylated forms of ERK 1 and 2, but it did not reach statistical significance (data not shown). Thus, resveratrol and genistein may exert chemoprotective actions on growth factor signaling molecules, and the coactivator molecules for the sex steroid receptors.
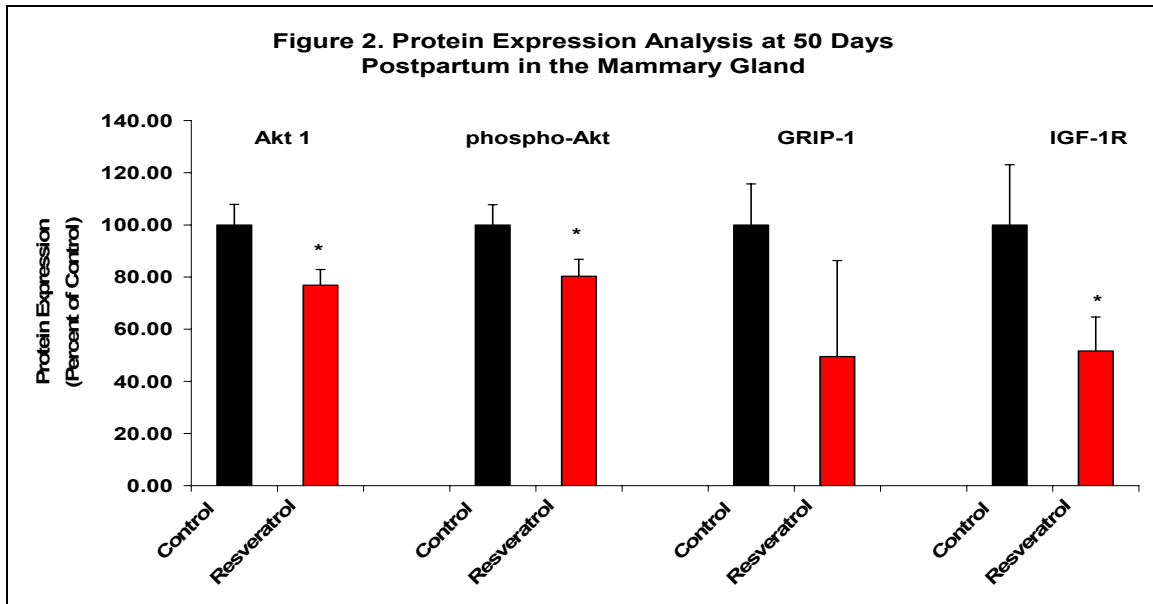
*Figure 2. Rats treated ± resveratrol (1000mg/kg diet) were sacrificed at 50 days postpartum. Mammary glands were dissected and assayed for protein expression by western blot analysis. Results are given as means + SEM, with the Control set to 100. * represents a p value < 0.05*

The combination of genistein and resveratrol treatments did not reveal any changes that were not observed in either of the single administrations (data not shown). This fits in with our recent data that showed combinations of genistein and resveratrol were protective against mammary carcinogenesis, but not any better than either agent by itself [8].

Aim 2. (Months 12-36). The goal of this aim was to investigate if genistein and resveratrol act independently of the estrogen receptors. This was carried out in bilaterally ovariectomized rats to eliminate endogenous ovarian estrogens and the use of ICI 182,780 to block estrogen receptors-alpha and beta (ER-α and β). Estradiol benzoate (EB) was used as a positive, estrogenic control.

The fourth abdominal mammary glands and the uteri were dissected from rats that had been ovariectomized at 7 weeks of age and injected with a subcutaneous dose of genistein

(500 mg/kg BW), resveratrol (500 mg/kg BW), estradiol benzoate (500 ug/kg BW), or vehicle control (equivalent volume of dimethyl sulphoxide (DMSO)) after three weeks of recovery (10 weeks of age). The subcutaneous dose followed pre-treatment ± the pure antiestrogen, ICI 182,780 (4 mg/kg BW) to block the estrogen receptors. A look at the uterine to body weight ratios (Table 1) demonstrates the effectiveness of the ICI 182,780 treatments. This is especially evident with the estradiol benzoate injection with or without ICI 182,780 pretreatment. Without ICI 182,780 pretreatment, EB resulted in a statistically significant increase (74% compared to the control group) in the uterine to body weight ratio, suggesting uterine growth and proliferation as would be expected with an estrogenic agonist. Blocking the estrogen receptors by ICI 182,780, the uterine to body weight ratio was not significantly different from the control group following EB injection. We would have expected the ICI + EB group to result in a greater reduction in uterine to body weight ratio (more similar to the control group), and thus may increase the dose of ICI 182,780 in future experiments. It should be noted that genistein and resveratrol had no statistically significant effect on uterine to body weight ratios, although genistein-treated rats displayed a 34% increase compared to the control group. This would fit with the characterization of genistein as a weak estrogen agonist. As one would expect, the uterine to body weight ratio of the Sham group (ovary intact) was significantly increased compared to any rat that was ovariectomized.

Treatment	Uterine to Body Weight Ratio	Percent of Control
Sham (ovary intact)	2.07 ± 0.23 *	581
Control (DMSO)	0.36 ± 0.02	100
Estradiol Benzoate (EB)	0.62 ± 0.09 *	174
Genistein	0.48 ± 0.11	134
Resveratrol	0.39 ± 0.04	110
ICI + Control	0.34 ± 0.02	96
ICI + EB	0.48 ±0.02	134
ICI + Genistein	0.38 ± 0.03	106
ICI + Resveratrol	0.31 ± 0.03	87

Table 1. Ovariectomized rats were injected subcutaneously with polyphenols or estrogen with or without pretreatment with ICI 182,780. Uteri were dissected and weighed to obtain uterine to body weight ratios.
* represents a p value < 0.05 as compared to the Control group.

As mentioned above, the fourth abdominal mammary gland was removed from rats that had been ovariectomized to remove endogenous estrogens, pretreated ± the antiestrogen ICI 182,780, and then treated with a single, subcutaneous dose of estradiol benzoate, genistein, or resveratrol. I will discuss the protein expression results for each estrogen or phytoestrogen individually.

Estradiol benzoate. As seen in Table 1, a single subcutaneous dose of estradiol benzoate significantly increased the uterine to body weight ratio compared to the Control (DMSO) group. We expected to see a significant decrease in protein levels of ER α and β along with an increase in progesterone receptor (PR) levels. We and others have shown that

activation of the estrogen receptors by estradiol causes a reduction in receptor levels hypothesized to be associated with receptor ubiquitin-proteosomal degradation [9]. An increase in mRNA and protein levels of PR is one of the classical responses to ER activation due to an estrogen response element in the progesterone receptor gene promoter region. We expected all of these responses to be blocked by pretreatment with ICI 182,780. Surprisingly, we observed no changes in the estrogen receptor protein levels (although the trend for ER β was down-regulation). We also observed no change in PR protein expression levels. One explanation for these observations is that these actions (decreased ER and increased PR) may have already occurred by our sacrifice time of 16 hours after estrogen exposure. Another possibility is that the activity levels of the receptors are modulated with no change at this time point in receptor protein levels. We are currently running these blots again to ensure that our observations were accurate. We observed no significant change in the steroid receptor coactivators (SRC-1, GRIP-1, or amplified in breast cancer 1 (AIB1)). Treatment with EB tended to increase the levels of EGFR, an event that was not abrogated by pre-treating the rats with ICI 182,780. This confirms the possibility that estrogens can affect growth factor pathways through non-estrogen receptor pathways. Interestingly, we observed increases in both the phosphorylated (active) forms of Akt and ERKs1&2 (Figure 3), although the results did not reach statistical significance. These increases may help to explain the estrogenic growth effects observed in the uterus. The actions on phospho-Akt and phospho-ERKs 1&2 were abrogated with pretreatment of ICI 182,780, suggesting a mechanism that involves the estrogen receptors.
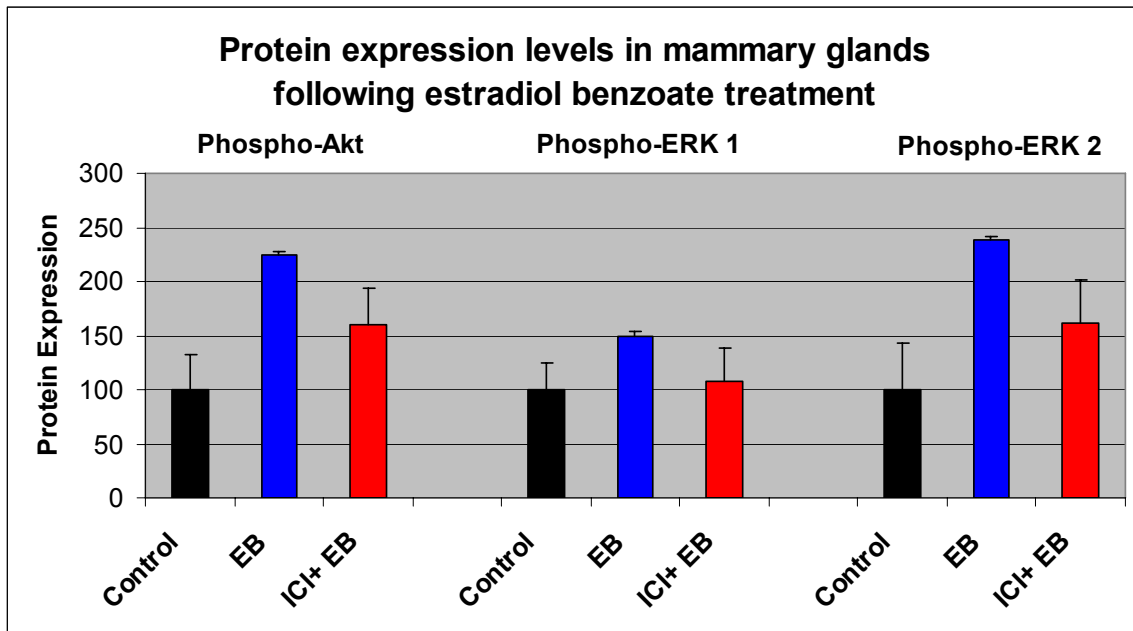

Figure 3. Ovariectomized rats treated ± ICI followed by EB were sacrificed. Mammary glands were dissected and assayed for protein expression by western blot analysis. Results are given as means + SEM, with the Controls set to 100.

Genistein. The effect of removing endogenous estrogens, using subcutaneous injection as the route of administration, and a pharmacologic dose of genistein (500 mg/kg BW) had the greatest effect on the actions of genistein in the rat mammary gland, compared to those effects seen after dietary exposure. The significant decreases in SRC-1 and GRIP-1 that we observed following dietary administration (Figure 1) were not present in this ovariectomized model following genistein injection. All three steroid receptor coactivators tested (SRC-1, GRIP-1, and AIB1) tended to have increased protein expression compared to the control group, although none of the three reached statistical significance (data not shown). Interestingly, pretreatment with the antiestrogen ICI 182,780 resulted in levels more similar to the control group, implicating that genistein may have actions through the estrogen receptors. The affinity of genistein binding to ER-beta has previously been reported [10]. To this point, we expected a reduction in the protein levels of ER-alpha and beta as shown previously [9]. Protein levels of ER-alpha and -beta were unchanged compared to the control group. As with estradiol benzoate treatment, we may have missed this action at the 16 hour time point that was used in this study. We also observed no significant differences in the protein levels of IGF-1R, the phosphorylated forms of Akt and ERKs 1 & 2, PR, or total ERK1s & 2 in this model, similar to what we reported after the administration of genistein in the diet (Aim1). These data point towards the critical importance of the route of administration and dose of genistein used for elucidating mechanisms of action.

Resveratrol. In this model (ovariectomy and subcutaneous injections), resveratrol caused protein expression changes that may elucidate the mammary chemoprotective effects that have been previously observed [4-6 and 8]. Exposure to resveratrol resulted in a statistically significant reduction in both ER α and PR b (Figure 4). Pretreatment with ICI 182,780 abrogated both of these protein modulations. Both the estrogen and progesterone receptors are known to play a role in mammary gland growth, proliferation, and disease progression and a decrease may help to explain the protective mechanisms of resveratrol. SRC-1 was also down-regulated by resveratrol treatment, though it did not reach statistical significance (data not shown). This effect was not blocked by ICI 182,780 pre-treatment suggesting a non-ER mediated effect. A decrease in this coactivator might also help to explain the protective effects of resveratrol as the coactivators allow for more efficient transcriptional activity of the steroid receptors such as ER and PR. We observed no change in the levels of co-activators AIB1 and GRIP-1 in this model (data not shown).
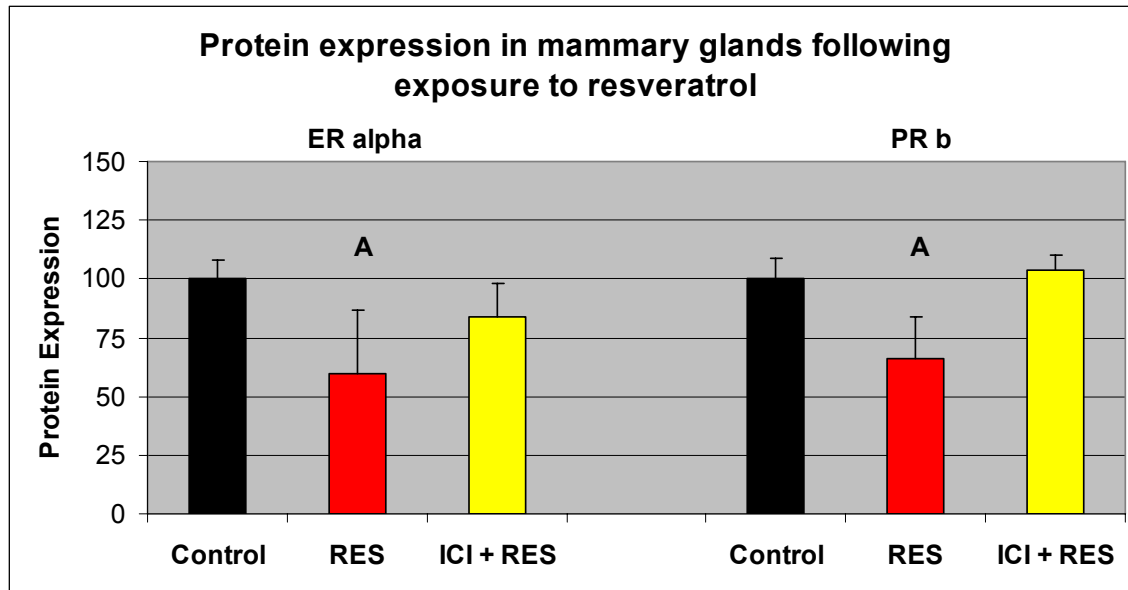
Figure 4. Ovariectomized rats treated ± ICI followed by resveratrol treatment were sacrificed. Mammary glands were dissected and assayed for protein expression by western blot analysis. Results are given as means + SEM, with the Controls set to 100. A represents a p value < 0.05

We also observed changes in growth factors that are known to play a role in mammary development and disease. EGFR protein expression was significantly decreased following resveratrol injection (Figure 5). This action was inhibited by pretreatment with the antiestrogen, ICI 182,780. A statistically significant decrease in the phosphorylated (active) forms of Akt and ERKs 1&2 was also observed (Figure 5). It should be noted that this is the opposite effect observed following EB treatment (increased phospho-Akt and ERKs 1&2 protein levels). This effect was also seen in Aim 1 (Figure 2) with the administration of resveratrol in the diet. Interestingly, pretreatment with a pure antiestrogen (ICI) resulted in no change in the protein levels of phospho-Akt and ERKs 1&2. This suggests a role for the estrogen receptors in the mechanisms observed for resveratrol. Cross-talk between the estrogen receptor and several growth factor pathways (EGF and IGF pathways) have been suggested *in vitro* and certainly warrant further *in vivo* investigation in the chemopreventive mechanisms of resveratrol. We observed no differences in the protein expression of total ERK 1&2 (data not shown).
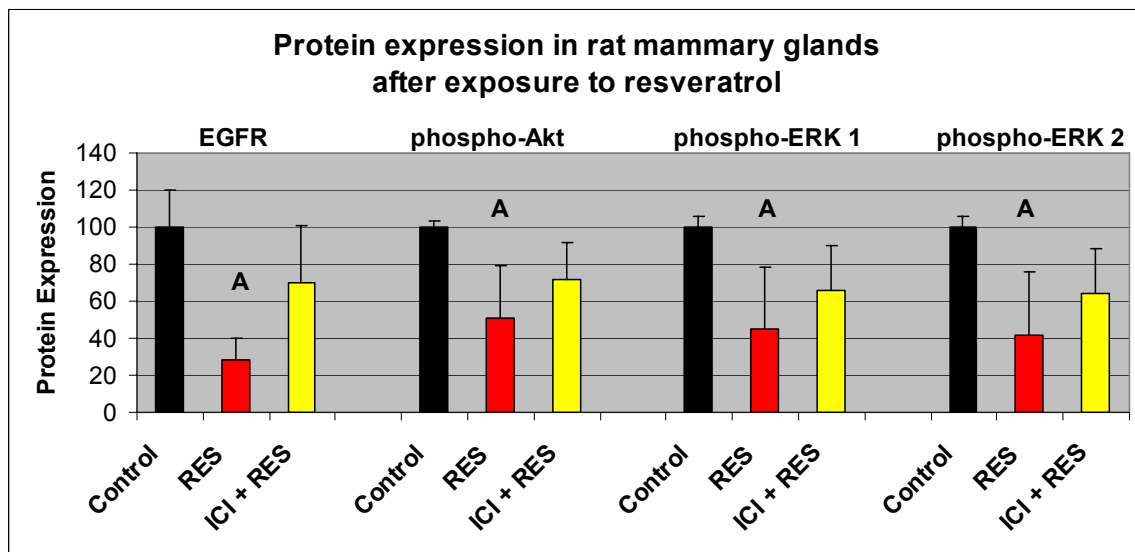
Figure 5. Ovariectomized rats treated ± ICI followed by resveratrol treatment were sacrificed. Mammary glands were dissected and assayed for protein expression by western blot analysis. Results are given as means + SEM, with the Controls set to 100. A represents a p value < 0.05

Aim 3. (Months 24-36). The goal of this revised aim was to further elucidate mechanisms through which resveratrol and genistein can protect against mammary carcinogenesis in an *in vivo* model. We employed TaqMan Low Density Arrays (TLDA), which allowed us to measure message levels (RNA) of 96 unique genes simultaneously by quantitative, real-time PCR. The 96 genes (Appendix A, Table 2) were chosen based on known roles in mammary gland growth, development, proliferation, apoptosis, and carcinogenesis.

The fourth abdominal mammary gland was dissected at 50 days postpartum after treatment with Control (AIN-76A), resveratrol (1 g/kg diet), or genistein (250 mg/kg diet) from birth. Mammary glands were frozen and then crushed in liquid nitrogen. RNA was isolated from the mammary glands using the RiboPure kit (Ambion, Austin, TX) as per the manufacturer's instructions. RNA was converted into cDNA and loaded in the arrays. The arrays simultaneously run quantitative, real-time PCR on 96 genes that were selected. 18s was used as an endogenous control gene. Several genes that could play a role in the chemoprevention observed with resveratrol and genistein were modulated in the mammary gland.

As shown in Figures 6, 7, and 8, exposure to resveratrol in the diet at 1 g/kg diet resulted in significant modulation of genes involved in differentiation, inflammation and apoptosis. Figure 6 shows that treatment with resveratrol resulted in a significant down-regulation of cyclooxygenase-1 (COX-1) and a trend (p value = 0.07) towards increased expression of peroxisome proliferator-activated receptor-gamma (PPAR-γ). COX-1 is

one of the rate limiting enzymes, along with COX-2, in the production of prostaglandins. As such, the COX enzymes are involved in inflammation and are often up-regulated in cancer, including cancers of the breast [11]. PPAR-γ is another receptor that has received attention in the breast cancer research field. The PPAR members play a key role in controlling cell differentiation, proliferation, inflammation and apoptosis [12]. PPAR-γ is expressed in many cancers, including breast and prostate cancers. In breast cancer cells, activation of PPAR-γ caused growth arrest and terminal differentiation [13]. This same report demonstrated that PPAR-γ ligands could inhibit the development of rat mammary carcinogenesis. Here we demonstrated that exposure to resveratrol in the diet may increase the gene expression of PPAR-γ in the mammary gland compared to the control group. Interestingly, reports have shown that blocking the COX enzymes and activating PPAR-γ caused an inhibition of breast cancer cell proliferation and increased apoptosis.
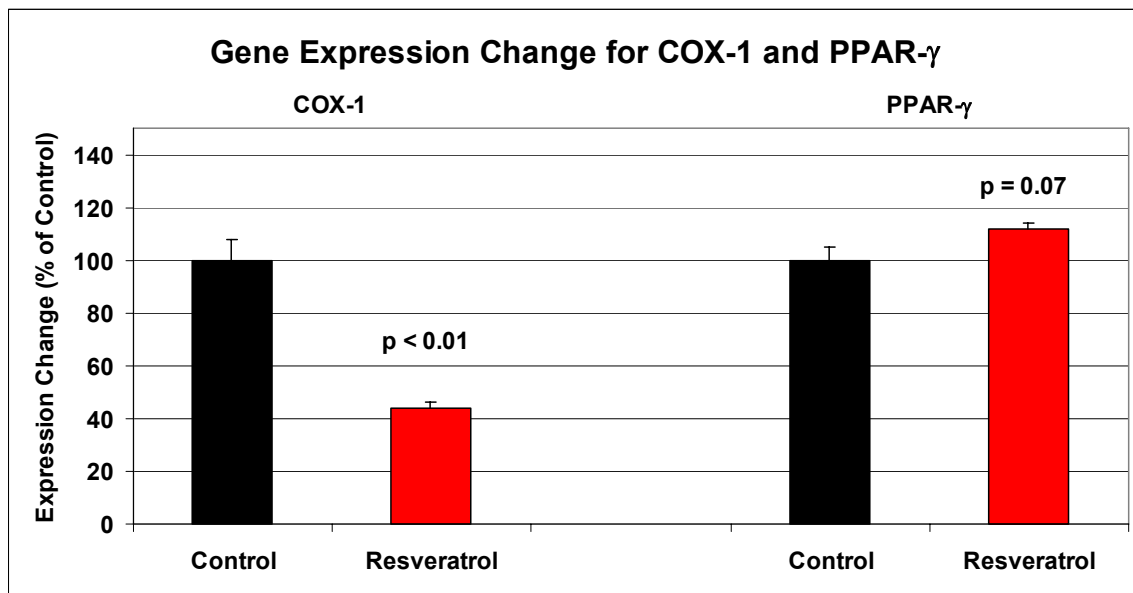

Figure 6. Gene expression changes in COX-1 and PPAR-γ in the mammary gland after exposure to resveratrol in the diet. TaqMan low density arrays were performed to measure gene expression by quantitative, real-time PCR. Female Sprague-Dawley rats were exposed to 1 g resveratrol/kg AIN-76A diet or AIN-76A diet from birth through 50 days postpartum. Values represent an average delta C_T value ± SEM, shown as a percentage of the control group. The direction of the expression change has been inverted as an increase in delta C_T value corresponds to a decrease in gene expression. A p value ≤ 0.05 was considered statistically significant.

As shown in Figure 7, treatment with resveratrol in the diet had a significant effect on the RNA expression of several members of the caspase family of enzymes. Caspases are critical components in the apoptotic cascade and caspases 2, 3 and 9 are directly involved in apoptosis, whereas other caspases are involved in cytokine processing [14]. Caspases 2 and 9 are 'initiator' caspases that serve to activate other caspases and amplify the apoptotic signal. Caspase 3 is an 'effector' caspase that acts to cleave DNA and ultimately kill the cell. Increases in the mRNA expressions of caspases-3 and -9 have

been associated with increased apoptosis in human mammary cancer cells [15]. Here we show that resveratrol treatment resulted in an increase in the gene expression of caspase 2, caspase-9 and caspase-3. Increased message level was demonstrated by a significant decrease in caspases 2 and 3, and a trend (p value = 0.10) for decreased expression of caspase 9. Increased expression of these caspase enzymes may play a role in the differences that we observed in epithelial cell apoptosis after exposure to resveratrol [6,8].
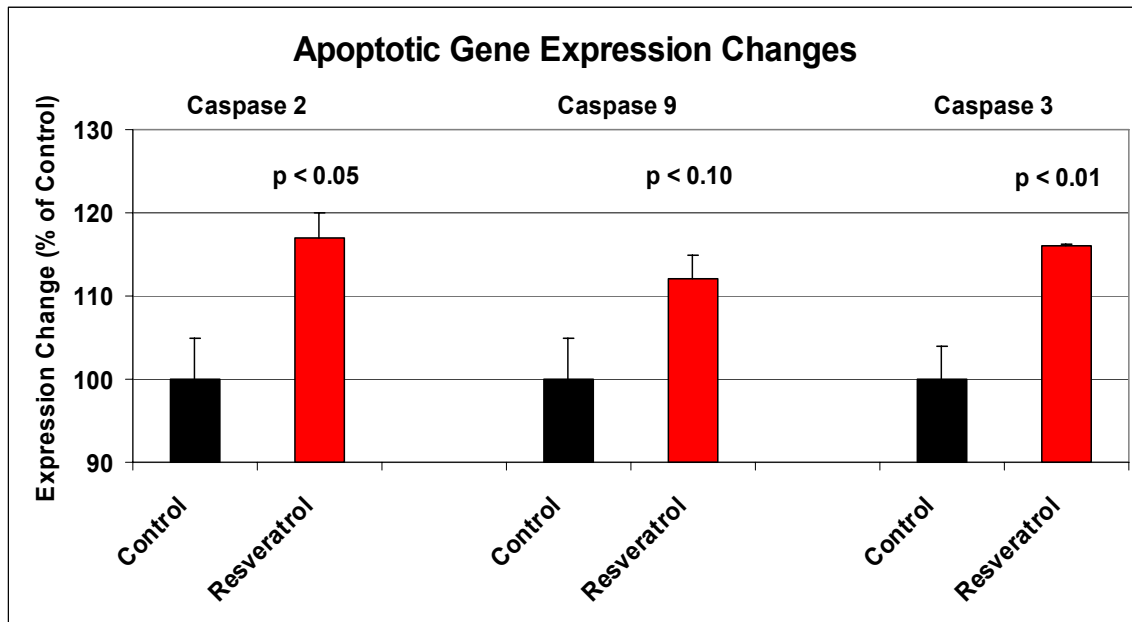

Figure 7. Apoptotic gene expression changes in mammary glands of rats exposed ± resveratrol in the diet. TaqMan low density arrays were performed to measure gene expression by quantitative, real-time PCR. Female Sprague-Dawley rats were exposed to 1 g resveratrol/kg AIN-76A diet or AIN-76A diet from birth through 50 days postpartum. Values represent an average delta C_T value ± SEM, shown as a percentage of the control group. The direction of the expression change has been inverted as a decrease in delta C_T value corresponds to an increase in gene expression. A p value ≤ 0.05 was considered statistically significant.

Treatment with resveratrol or genistein in the diet significantly modulated only one common gene, beta casein. Both treatments resulted in an increase in gene expression, compared to the control group (Figure 8). Beta casein is a milk protein and a biomarker of mature mammary glands and differentiated cells in the breast. The increased gene expression of beta casein associates well with mammary whole mount data showing that resveratrol [6] and especially genistein [3] can increase the number of mature lobular structures in the mammary gland. It was previously reported that prepubertal exposure to genistein in the diet resulted in a 6-fold increase in beta casein protein levels in the mammary gland [reviewed in 7]. This increase in beta casein and mammary maturation may play a role in the chemoprevention that was observed for each of these polyphenols.
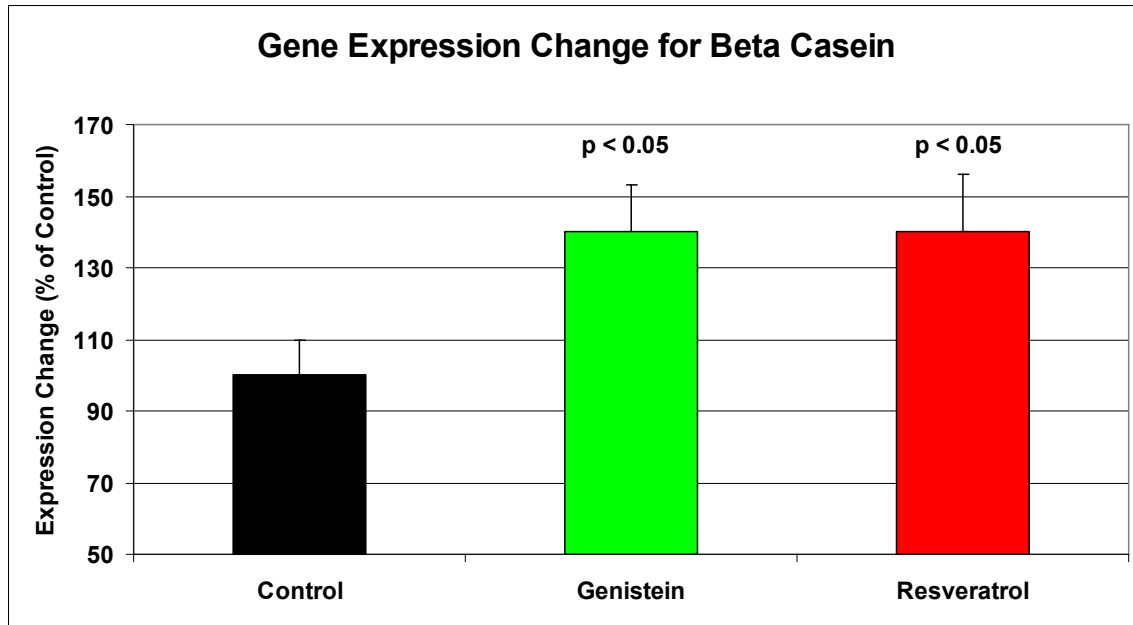
Figure 8. Gene expression analysis of beta casein in the mammary gland after exposure to genistein or resveratrol in the diet. TaqMan low density arrays were performed to measure gene expression by quantitative, real-time PCR. Female Sprague-Dawley rats were exposed to 1 g resveratrol/kg AIN-76A diet, 250 mg genistein/kg AIN-76A diet, or AIN-76A diet from birth through 50 days postpartum. Values represent an average delta C_T value $\pm$ SEM, shown as a percentage of the control group. The direction of the expression change has been inverted as a decrease in delta C_T value corresponds to an increase in gene expression. A p value ≤ 0.05 was considered statistically significant.

Key Research Accomplishments:

- Significant modulation of several proteins by genistein and resveratrol was detected in Aim 1. Many of these are important for mammary growth, proliferation, and chemoprevention by genistein and resveratrol at 50 days postpartum.

- The use of ovariectomy and blocking of the estrogen receptors were effective as evidence by uterine to body weight ratio changes. In this model, estradiol benzoate was able to modulate growth factor pathways. Resveratrol was able to affect both sex steroid pathways and growth factor paths that are involved in mammary gland development and carcinogenesis.

- Based on data from Aims 1 and 2, we believe that the timing of exposure, the route of administration, and the dose of genistein treatment can significantly impact the mechanism of action in the mammary gland. The protective effects that were observed after dietary administration (decreased levels of SRC-1 and GRIP-1) were not observed after pharmacologic injection in ovariectomized rats.

-   The use of Taqman Low Density Arrays (TLDA) was introduced to our lab and used to measure 96 genes by quantitative, real-time PCR simultaneously.

-   As measured by TLDA, resveratrol was shown to regulate COX-1 and PPAR-g, both of which have been reported to play a role in mammary carcinogenesis. A decrease in COX-1 and increase in PPAR-g may help to explain the protection against mammary cancer.

-   Resveratrol also increased the message levels of three caspases, and elucidates mechanisms by which resveratrol increases apoptosis in the mammary gland.

-   The TLDA analysis showed that both resveratrol and genistein up-regulated beta casein, a marker a mammary gland maturation. This increased maturation may help to explain how these polyphenols protect against carcinogenesis.

-   A manuscript to the Journal of Carcinogenesis (2006) was accepted dealing with the chemopreventive properties of resveratrol. This work has become one of the ten most viewed papers of the past year in the journal.

-   Data and ideas stemming from this grant were used to publish a review article in Expert Reviews in Anticancer Therapies (2006).

-   Data from this project was used to attend a conference and present a poster at the 2005 Gordon Research Conference: Hormonal Carcinogenesis. A graduate student fellow award was received for the work.

-   Data from this project was used to attend and present a poster at the 2005 Society of Toxicology national meeting. A graduate student travel award was received for the work.

-   Data from this project was used to attend and present a poster at the 2005 and the 2006 UAB Comprehensive Cancer Center Annual Research Retreat. The PI (Tim Whitsett) received the prestigious William Bailey Award for Excellence in Cancer Prevention and Control for this work.

-   Data from this project was accepted for poster presentation for two separate meetings in 2006: AACR annual meeting and the Gordon Research Conference: Mammary Gland Biology.

-   Some of the data obtained from this project were used to help the PI (Tim Whitsett) qualify into candidacy in the UAB Pharm/Tox doctoral program.

-   Much of the data generated from this grant were used in the PI's doctoral dissertation, satisfying the doctoral degree requirement for a Ph.D. in the Dept. of Pharmacology and Toxicology at the University of Alabama at Birmingham.

- Data from this grant was presented at a Postdoctoral interview which led to a Postdoctoral Fellowship at the Translational Genomics Research Institute.

Reportable Outcomes:

PUBLICATIONS (See Appendix C and Appendix D for full publications):

Whitsett T, Lamartiniere CA. Genistein and resveratrol: mammary cancer chemoprevention and mechanisms of action in the rat. *Expert Rev. Anticancer Ther.*, 6(12), 2006.

Whitsett T, Carpenter DM, Lamartiniere CA. Resveratrol, but not EGCG, in the diet suppresses DMBA-induced mammary cancer in rats. *J. Carcinog*. 5:15, 2006.
This publication was one of the 10 most-viewed papers in this journal in 2007.

Some of the results from this grant have been used in poster presentations at national and international scientific meetings over the past two years.

ABSTRACTS (See Appendix D for complete absract):

Whitsett T, Chen D, Lamartiniere C. Mammary cancer chemoprevention with the polyphenols genistein and resveratrol. Third Annual Early Environmental Exposures Meeting, Breast Cancer and Environment Research Centers. San Francisco, CA. Nov. 2006.

Whitsett T, Chen D, Lamartiniere C. Mammary cancer chemoprevention with the polyphenol resveratrol. Gordon Research Conference: Mammary Gland Biology. Lucca, Italy. June 2006.

Whitsett T, Chen D, Lamartiniere C. Mammary cancer chemoprevention with the polyphenol resveratrol. AACR Annual Meeting. Proceedings, Volume 47, p. 534. Washington D.C. April 2006.

Whitsett T and Lamartiniere CA. Breast Cancer Chemoprevention with the Polyphenol Resveratrol. Gordon Research Conference: Hormone Action in Development and Cancer. Holyoke, MA. July 2005.
A graduate student fellow award was received for the work.

Whitsett T and Lamartiniere CA. Breast cancer chemoprevention with the polyphenol resveratrol. Society of Toxicology 44 Annual Meeting. Program page 164. New Orleans, LA. March 2005.
A student travel award was received for the work.

The data was also used in an invited seminar at the University of Alabama at Birmingham. The title of the seminar was: "Mammary Cancer Chemoprevention with the

Polyphenols Genistein and Resveratrol" UAB Pharmacology and Toxicology Seminar, Feb. 2006

The data from this grant was also used in an invited lecture: "Nutrigenomics: Driving Health by Nutrition" A pre-conference short course for 2007 Beyond Genome Conference, San Francisco, CA, June 2007. The title of the lecture was: "Breast Cancer Chemoprevention with the Natural Polyphenols Genistein and Resveratrol".

Much of the data generated from this grant were included in the dissertation of the PI (Timothy Whitsett) entitled: **BREAST CANCER CHEMOPREVENTION WITH THE NATURAL POLYPHENOLS RESVERATROL AND GENISTEIN, ALONE AND IN COMBINATION** and satisfied the requirements for conferring a doctoral degree in the Dept. of Pharmacology and Toxicology at the University of Alabama at Birmingham.

Conclusions:

We and others have clearly shown a protection against mammary carcinogenesis using the polyphenols genistein and resveratrol in the diet. We have also shown the ability of these polyphenols to modulate mammary gland maturation, as well as cell proliferation and apoptosis. This grant aimed to look at the molecular level to elucidate the mechanisms through which these polyphenols act. Through year two of the project, we have shown that genistein and resveratrol can regulate important molecules in both the growth receptor pathways and the sex steroid receptor pathways at 50 days postpartum. We have now begun to look at the importance of the estrogen receptors in the mechanisms of genistein and resveratrol. Estradiol benzoate was used as a positive estrogenic control and resulted in a significant increase of uterine to body weight ratio. The growth effects may involve the Akt and ERK pathways. Resveratrol, in the ovariectomized model, was able to affect both sex steroid pathways, as well as growth pathways such as EGF and Akt pathways. To further elucidate the molecular changes that could account for the mammary chemoprevention, cell proliferation, and apoptosis that we observed, we employed Taqman low density arrays. This technique allowed for the detection of 96 unique gene expression changes by quantitative, real-time PCR simultaneously. We designed an array which looked at genes that are thought to play a role in mammary gland development, apoptosis, carcinogen activation and mammary carcinogenesis. We report that treatment of resveratrol in the diet caused a significant decrease in the gene expression of COX-1 and a decrease in gene expression of PPAR-γ. Inhibition of COX enzymes and activation of PPAR receptors have previously been shown to induce breast cancer cell proliferation and inhibit proliferation. These gene modulations help to explain the changes that that we observed in mammary gland cell proliferation and apoptosis. In the low density array, we also show a significant increase in members of the caspase enzyme family. Resveratrol treatment resulted in increased gene expression of the initiator caspases 2 and 9 and the executioner caspase 3. These critical components of the apoptotic cascade may help to explain the differences that we observed in mammary gland apoptosis. There is much more to learn about the

mechanisms of these polyphenols so that they may be used in clinical trials and help women to reduce their risk of breast cancer.

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APPENDEX A

Table 2. TaqMan Low Density Array: 96 Unique Genes Selected for Quantitation

Gene Name	Gene Symbol	Category
Cytochrome P450 1a1	Cyp1a1	Cytochrome P450
Cytochrome P450 1a2	Cyp1a2	Cytochrome P450
Cytochrome P450 1b1	Cyp1b1	Cytochrome P450
Cytochrome P450 2a1	Cyp2a1	Cytochrome P450
Cytochrome P450 2a2	Cyp2a2	Cytochrome P450
Cytochrome P450 4b1	Cyp4b1	Cytochrome P450
Cytochrome P450 11a1	Cyp11a1	Cytochrome P450
Cytochrome P450 17a1	Cyp17a1	Cytochrome P450
Cytochrome P450 19a1 (aromatase)	Cyp19a1	Cytochrome P450
Catechol-O-methyltransferase	Comt	Carcinogen Metabolism
		Genistein-regulated
GTP cyclohydrolase 1	Gch	
Caspase 2	Casp2	Apoptosis
Caspase 3	Casp3	Apoptosis
Caspase 6	Casp6	Apoptosis
Caspase 7	Casp7	Apoptosis
Caspase 8	Casp8	Apoptosis
Caspase 9	Casp9	Apoptosis
Bcl2-associated death promoter	Bad	Apoptosis
Bcl2-antagonist/killer 1	Bak1	Apoptosis
Bcl2-associated X protein	Bax	Apoptosis
B-cell leukemia/lymphoma 2	Bcl2	Apoptosis
Bcl2 related protein A1	Bcl2a1	Apoptosis
Bcl2-like 1 (Bcl-xl)	Bcl2l1	Apoptosis
Bcl2-like 2 (Bcl-W)	Bcl2l2	Apoptosis
BH3 interacting domain death agonist	Bid	Apoptosis
Fas ligand	Faslg	Apoptosis
Apoptotic peptidase activating factor 1	Apaf1	Apoptosis
Death effector domain-containing	Dedd	Apoptosis
Death-associated protein	Dap	Apoptosis
Defender against cell death 1	Dad1	Apoptosis
Estrogen receptor alpha	Esr1	Sex Steroid/Growth Factor
Estrogen receptor beta	Esr2	Sex Steroid/Growth Factor

Epidermal Growth Factor receptor (ErbB-1)	Egfr	Sex Steroid/Growth Factor
ErbB-2	Erbb2	Sex Steroid/Growth Factor
ErbB-3	Erbb3	Sex Steroid/Growth Factor
ErbB-4	Erbb4	Sex Steroid/Growth Factor
Androgen receptor	Ar	Sex Steroid/Growth Factor
Fibroblast growth factor 1	Fgfr1	Sex Steroid/Growth Factor
Hepatocyte growth factor	Hgf	Sex Steroid/Growth Factor
Insulin-like growth factor 1	Igf1	Sex Steroid/Growth Factor
Insulin-like growth factor 2	Igf2	Sex Steroid/Growth Factor
Insulin-like growth factor 1 receptor	Igf1r	Sex Steroid/Growth Factor
Insulin receptor	Insr	Sex Steroid/Growth Factor
VEGF receptor 2	Kdr	Sex Steroid/Growth Factor
Leptin	Lep	Sex Steroid/Growth Factor
Leptin receptor	Lepr	Sex Steroid/Growth Factor
Mitogen activated protein kinase 1 (Erk 2)	Mapk1	Sex Steroid/Growth Factor
Mitogen activated protein kinase kinase 1	Map2k1	Sex Steroid/Growth Factor
Mitogen activated protein kinase 3 (Erk 1)	Mapk3	Sex Steroid/Growth Factor
Nuclear receptor coactivator 2	Ncoa2	Sex Steroid/Growth Factor
Glucocorticoid receptor	Nr3c1	Sex Steroid/Growth Factor
Platelet-derived growth factor receptor	Pdgfrb	Sex Steroid/Growth Factor
Progesterone receptor	Pgr	Sex Steroid/Growth Factor
Peroxisome proliferators-activating receptor alpha	Ppara	Sex Steroid/Growth Factor
Peroxisome proliferators-activating receptor delta	Ppard	Sex Steroid/Growth Factor
Peroxisome proliferators-activating receptor gamma	Pparg	Sex Steroid/Growth Factor
Retinoic acid receptor alpha	Rara	Sex Steroid/Growth Factor
Retinoid X receptor alpha	Rxra	Sex Steroid/Growth Factor
Retinoid X receptor beta	Rxrb	Sex Steroid/Growth Factor
Vitamin D receptor	Vdr	Sex Steroid/Growth Factor
Vascular endothelial growth factor A	Vegfa	Sex Steroid/Growth Factor
Thyroid hormone receptor alpha	Thra	Sex Steroid/Growth Factor
Thyroid hormone receptor beta	Thrb	Sex Steroid/Growth Factor
Cyclin b1	Ccnb1	Cyclin/Proliferation

Cyclin d1	Ccnd1	Cyclin/Proliferation
Cyclin d3	Ccnd3	Cyclin/Proliferation
Cyclin g1	Ccng1	Cyclin/Proliferation
Janus kinase 2	Jak2	Cyclin/Proliferation
Janus kinase 3	Jak3	Cyclin/Proliferation
Signal transducer and activator of transcription 3	Stat3	Cyclin/Proliferation
Signal transducer and activator of transcription 5a	Stat5a	Cyclin/Proliferation
Myelocytomatosis viral oncogene homolog	Myc	Cyclin/Proliferation
Met proto-oncogene	Met	Cyclin/Proliferation
Thymoma viral proto-oncogene 1	Akt1	Akt Signaling
Thymoma viral proto-oncogene 2	Akt2	Akt Signaling
Thymoma viral proto-oncogene 3	Akt3	Akt Signaling
Phosphatase and tensin homolog	Pten	Akt Signaling
Breast cancer 1	Brac1	Mammary Cancer
Breast cancer 2	Brca2	Mammary Cancer
p53	Tp53	Mammary Cancer
Beta casein	Csn2	Mammary Maturity
Interleukin 6	Il6	Inflammation/Growth
Transforming growth factor alpha	Tgfa	Inflammation/Growth
Transforming growth factor, beta 3	Tgfb3	Inflammation/Growth
Transforming growth factor, beta receptor 1	Tgfbr1	Inflammation/Growth
Transforming growth factor, beta receptor 2	Tgfbr2	Inflammation/Growth
Cyclooxygenase-1	Ptgs1	Inflammation/Growth
Cyclooxygenase-2	Ptgs2	Inflammation/Growth
Tumor necrosis factor	Tnf	Inflammation/Growth
Matrix metalloprotease 9	Mmp9	Inflammation/Growth
Growth arrest and DNA damage-inducible 45 alpha	Gadd45a	Inflammation/Growth
NFκB	Rela	Inflammation/Growth
Inhibitor of kappa B kinase beta	Ikbkb	Inflammation/Growth
Eukaryotic 18S rRNA	18S	Endogenous Control
Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	Endogenous Control
TATA box binding protein	Tbp	Endogenous Control

APPENDIX B.

A pdf version of the published manuscript entitled: "Resveratrol, but not EGCG, in the diet suppresses DMBA-induced mammary cancer in rats."

Research

Resveratrol, but not EGCG, in the diet suppresses DMBA-induced mammary cancer in rats

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Abstract

Despite the advent of new and aggressive therapeutics, breast cancer remains a leading killer among women; hence there is a need for the prevention of this disease. Several naturally occurring polyphenols have received much attention for their health benefits, including anti-carcinogenic properties. Two of these are resveratrol, a component of red grapes, and epigallocatechin-3-gallate (EGCG), the major catechin found in green tea. In this study, we tested the hypothesis that these two polyphenols protect against chemically-induced mammary cancer by modulating mammary gland architecture, cell proliferation, and apoptosis. Female Sprague-Dawley CD rats were exposed to either resveratrol (1 g/kg AIN-76A diet), EGCG (0.065% in the drinking water), or control diet (AIN-76A) for the entirety of their life starting at birth. At 50 days postpartum, rats were treated with 60 mg dimethylbenz[a]anthracene (DMBA)/kg body weight to induce mammary cancer. Resveratrol, but not EGCG, suppressed mammary carcinogenesis (fewer tumors per rat and longer tumor latency). Analysis of mammary whole mounts from 50-day-old rats revealed that resveratrol, but not EGCG, treatment resulted in more differentiated lobular structures. Bromodeoxyuridine (BrdU) incorporation studies showed that resveratrol treatment caused a significant reduction in proliferative cells in mammary terminal ductal structures at 50 days postpartum, making them less susceptible to carcinogen insult. The epithelial cells of terminal end buds in the mammary glands of resveratrol-treated rats also showed an increase in apoptotic cells compared to the control or EGCG-treated rats as measured by a DNA fragmentation assay. At the given doses, resveratrol treatment resulted in a serum resveratrol concentration of 2.00 μM, while treatment with EGCG resulted in a serum EGCG concentration of 31.06 nM. 17β-Estradiol, progesterone, and prolactin concentrations in the serum were not significantly affected by resveratrol or EGCG. Neither polyphenol treatment resulted in toxicity as tested by alterations in body weights, diet and drink consumptions, and day to vaginal opening. We conclude that resveratrol in the diet can reduce susceptibility to mammary cancer, while EGCG in the drinking water at the dose used was not effective.

Background

Breast cancer remains a leading killer among cancers that affect women in the United States and around the world. It was estimated that in 2005, in the US alone, there were

211,240 new cases of female breast cancer and 40,410 deaths [1]. This remains a destructive disease despite the advent of new and aggressive therapeutics. It is widely accepted that environmental and dietary factors play a role in determining one's risk of breast cancer. There is an extensive and growing amount of work devoted to the possible links between diet and a reduction in the risk of breast cancer. Our lab has studied the effects of dietary exposure to genistein, the primary isoflavone component of soybeans. We have shown that genistein administered in neonatal, prepubertal, and a combination of neonatal and prepubertal periods followed by adult exposures can suppress chemically-induced mammary cancer in Sprague-Dawley rats [2-4]. Other dietary compounds that have received much attention for their health benefits, including anti-carcinogenic properties, are the naturally occurring polyphenols resveratrol and EGCG.

Resveratrol is a polyphenolic phytoalexin present in grape skins and red wine that has been shown to have antioxidant and anti-inflammatory properties. In 1996, Jang et al. reported that resveratrol could inhibit a number of cellular events associated with the initiation, promotion, and progression of cancer [5]. That report has been followed with a battery of *in vitro* investigations into the chemopreventive activity of resveratrol [6,7]. Also, there have been reports in which resveratrol has been shown to reduce the induction of chemically-induced breast cancer models. Bhat et al. administered resveratrol *via* gavage and showed an increase in tumor latency and a decrease in the total number of tumors in an N-methyl-N-nitrosourea-(NMU) induced mammary cancer model [8]. Dietary resveratrol also reduced incidence and multiplicity and extended the latency period in a DMBA-induced mammary cancer model [9], although this study used a small number of animals and what the authors called a DMBA dose "suboptimal to produce sufficient tumors". Our study used dietary administration and employed at least 30 animals per group and a dose of DMBA that was sufficient to cause 100% tumor incidence and resulted in an average of 8.5 tumors per rat in the control group.

The consumption of tea has been associated with a host of health benefits including the prevention of cancer [10]. The chemopreventive effects of tea have been attributed to the large amount of polyphenolic catechins present in tea. A cup of tea contains 30–40% catechins by dry weight, with EGCG being the most prevalent catechin [11]. These catechins are strong antioxidants, have been associated with a reduced risk of cardiovascular disease, and reported to have anti-carcinogenic effects on skin, lung, oral cavity, stomach, colon, pancreas, and breast cancers in animal models [10]. Gupta et al. showed green tea polyphenols can inhibit prostate tumors in a transgenic mouse model [12].

To our knowledge, this is the first study that simultaneously investigated the potential of purified resveratrol and chemically-synthesized EGCG throughout life in the diet to suppress chemically-induced mammary cancer initiated with concentrations of DMBA that result in adenocarcinomas. We also investigated the potential of resveratrol or EGCG *via* the diet to modulate mammary gland maturation, cell proliferation, and apoptosis in terminal mammary structures as mechanisms for cancer chemoprevention.

Methods

Animals

This study was approved by the University of Alabama at Birmingham Animal Use Committee. Female Sprague-Dawley CD rats (Charles River, Raleigh, NC) were housed in a climate controlled room with a 12 hour light/dark cycle (light on at 8:00 AM) in the UAB Animal Resources Facility and fed phytoestrogen-free AIN-76A diet (Harlan Teklad, Madison, WI). At birth, litters were placed on one of three diets: 1) AIN-76A with tap water as the control, 2) 1 gram resveratrol/kg AIN-76A diet with tap water, or 3) 0.065% EGCG in the drinking water with AIN-76A diet. Resveratrol (Xi'an Sino-Dragon Import & Export Co., China) was extracted from Rhizoma Polygoni Cuspidati and tested as 98% pure by HPLC. EGCG (a gift from Roche Fine Chemicals, Basel, Switzerland) was chemically-synthesized at 93% purity as tested by HPLC. The bottles used to administer all drinking fluids were an amber color to suppress EGCG degradation. EGCG was changed daily around 3 PM. Treatments were started at birth and continued throughout the life of the animals, with the rats having free access to both food and drink. The resveratrol and EGCG doses were chosen based on previous work by Bhat et al. and Gupta et al. [8,12]. Bhat reported an inhibition of carcinogen-induced mammary tumors by gavaging rats with 100 mg resveratrol/kg body weight. Hence, we calculated that a 200 gram rat eating 20 grams of the provided diet with 1 mg resveratrol/g diet would consume ~100 mg resveratrol/kg body weight. Gupta showed a reduction in prostate tumors in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model using a 0.1% solution of green tea polyphenols (62% of which was EGCG). Animals were weaned at 21 days postpartum. Body weights and food and drink consumptions were measured at 21 and 50 days postpartum. Onset of vaginal opening was recorded as a marker for sexual maturity.

Tumor induction

At 50 days postpartum, 94 female rats (30 Control, 30 Resveratrol, and 34 EGCG) were gavaged with 60 mg dimethylbenz[a]anthracene (DMBA)/kg body weight, a dose sufficient to cause 100% tumor incidence in the control group over the course of the study. The DMBA was

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dissolved in sesame oil at a stock solution of 30 mg/ml. Animals were palpated twice a week starting five weeks after DMBA administration in order to record the presence, location, size, and date of detection for all tumors. Animals were sacrificed when the tumor diameter reached one inch, animals became moribund, or rats reached 18 weeks post-DMBA treatment. Sections from the tumors were fixed in 10% neutral buffered formalin and embedded in paraffin. Tumor blocks were sectioned and fixed on slides to be evaluated histopathologically by Dr. I. Eltoum, a board certified pathologist at UAB.

Mammary gland differentiation
Whole mounts of the fourth abdominal mammary glands were prepared from 50-day-old rats as previously described [2]. Briefly, mammary glands were removed at time of sacrifice and placed on a microscope slide. Glands were fixed in neutral buffered formalin followed by de-fatting in acetone. The glands were stained using 0.2% alum carmine (Sigma, St. Louis, MO). Slides were dehydrated in a series of graded alcohols from 35–100%. The slides were cleared in xylene and compressed between two glass slides. The glands were allowed to expand before being mounted using glass coverslips. Glands were analyzed using a Nikon light microscope (Nikon, Melville, NY) for the quantity of terminal ductal structures including: end buds, ducts, and lobules types I and II as previously described [2,3,13]. A terminal end bud was characterized as an elongated ductal structure with 3 to 6 epithelial cell layers and >100 micrometers in diameter. Terminal ducts have one to three epithelial cell layers and were <100 micrometers in diameter, while lobules I and II have 5–10 and 10–20 alveolar buds, respectively.

Cell proliferation
Bromodeoxyuridine (BrdU) (Sigma) incorporation was used as an index of cell proliferation. BrdU was administered two hours before sacrifice, injected i.p. with 100 mg BrdU/kg body weight. The contralateral fourth mammary gland was removed and fixed in 10% neutral buffered formalin. The glands were then placed in 70% ethanol overnight before being embedded in paraffin. The blocks were sectioned onto microscope slides. Slides were analyzed as previously done in our lab [3]. Briefly, paraffin was removed by xylene and the slides were rehydrated in ethanol. Slides were placed in 3.5 N HCl and then trypsinized. Slides were placed in 3% H_2O_2 (Sigma) to quench endogenous peroxidase and blocked in 10% normal horse serum (Vector, Burlingame, CA). Slides were incubated with monoclonal anti-BrdU primary antibody (Sigma) and subsequently incubated with biotinylated horse anti-mouse secondary antibody (Vector). Detection was performed using streptavidin (ABC reagent, Vector) and the color developed by DAB (Vector). Slides were counterstained with Gills no. 2 hematoxylin and then

dehydrated, cleared, and coverslipped. The labeling index, which is the number of cells incorporating BrdU divided by total number of cells × 100, was determined for terminal end buds, terminal ducts, and lobules types I and II using a Nikon light microscope and Nikon digital camera, and analyzed using Image J software (NIH).

Apoptosis assay
The TdT-FragEL™ DNA Fragmentation Detection Kit (Calbiochem, San Diego, CA) was used to measure apoptosis following the manufacturer's instructions. Briefly, paraffin-embedded tissue sections were deparaffinized and rehydrated in graded alcohols. Tissues were permeablized with Proteinase K and rinsed with Tris-Buffered Saline. Endogenous peroxidases were inactivated with 3% hydrogen peroxide and labeled with TdT enzyme. The labeling reaction was terminated with Stop Buffer. The tissues were blocked and then color detection was done with diaminobenzidine (DAB). The slides were couterstained with methyl green, dehydrated in alcohol and xylene, and coverslipped. The apoptotic index was the number of epithelial cells stained positive for apoptosis divided by the total number of epithelial cells counted in both mammary terminal end buds and lobule structures. Visualization was performed using a Nikon light microscope, Nikon digital camera, and analyzed using Image J software (NIH).

Serum polyphenol concentrations
Blood was collected from 50 day old rats at the time of sacrifice. Whole blood was spun at 2300 rpm for 15 minutes to collect serum and frozen at -80°C until use. Resveratrol and EGCG were extracted from the serum and measured on a 4000 Q TRAP® LC/MS/MS System (Applied Biosystems, Foster City, CA). Serum extractions were done as previously described [[3] and [14]]. For resveratrol, internal standards (apiginin, 4-methylumbelliferone, and phenolphthalein glucuronide) were added to serum followed by incubation with 1 mg β glucuronidase/sulfate enzyme (Sigma) in 250 uL ammonium acetate buffer. Samples were extracted in hexane and followed by ether extraction. Samples were redissolved in 80% methanol prior to LC/MS/MS analysis. For EGCG serum analysis, 1% acetic acid in 100% methanol was used after incubation with internal standards and β-glucuronidase enzyme. A separate standard curve was run for each experiment. Control serum was used as a negative control for both resveratrol and EGCG extractions.

Serum concentrations of 17β-estradiol, progesterone, and prolactin
Serum estradiol-17β, progesterone, and prolactin concentrations were measured using radio-immunoassays (Diagnostic Systems Laboratories, Webster, TX) as described by the manufacturer. All samples were run in duplicate.

Statistics

The time-to-event data, e.g., time-to-first-tumor (latency) and time-to-sacrifice (tumor burden), were analyzed using the LIFETEST and LIFEREG procedures in SAS® [15]. Survival functions were first estimated nonparametrically using Kaplan-Meier (KM) and compared across the three groups using the log-rank test and parametrically using Weibull regression analysis. Those animals that did not demonstrate the event in question by the end of study or before sacrifice were treated as censored in the time-to-event analysis and the end of study or sacrifice time substituted in for their actual times. Tumor multiplicity data were analyzed with the GENMOD (generalized linear models) procedure in SAS using generalized Poisson regression on the tumor appearance rates (assuming a negative binomial distribution). The rate of tumor appearance was computed for each animal as the total number of tumors divided by the number of days on the study. For more detail about generalized linear models and associated maximum likelihood estimation, see McCullagh and Nelder [16]. All biochemical measurements were done using Students t test with a p value of 0.05 as statistically significant, while group comparisons were analyzed by one way analysis of variance (ANOVA) in SAS.

Results

Body and uterine weights, and vaginal opening

Lifetime treatments with 1 g resveratrol/kg diet or 0.065% EGCG in the drinking water did not result in a significant difference in body weights as assessed at 21 or 50 days postpartum (Table 1). Likewise, there was no difference in the uterine/body weight ratio between the groups at ages 21 or 50 days. The average number of days to vaginal opening, a marker for sexual maturation, was not altered by resveratrol (34.95 ± 0.35) or EGCG (35.04 ± 0.35) treatment as compared to the control rats (34.16 ± 0.40) There were no observed differences in food or drink consumption between the control and either polyphenol treatment in 21, 35, 50, or 100 day old rats (data not shown).

Tumor studies

Female Sprague-Dawley rats exposed to resveratrol in the diet throughout life and to DMBA on day 50 postpartum had significantly lower tumor multiplicity rates (Figure 1) and significantly longer tumor latency (Figure 2A, B, and 2C) compared to control rats receiving DMBA. Animals exposed throughout life to EGCG in the drinking water showed a decrease in the latency to first tumor development, although there was no significant difference as compared to the control group with respect to second and third tumor latency (Table 2A, B, and 2C). Likewise, tumor multiplicity in EGCG-exposed rats was not significantly different from the controls. Animals receiving resveratrol developed the lowest number of chemically-induced mammary tumors per rat (4.39 ± 0.61) (Figure 1). This was a 50% reduction in the number of mammary tumors compared to control animals (8.79 ± 1.33; p < 0.001). At the time of necropsy, animals that received EGCG in the drinking water had fewer mammary tumors per rat than the controls (6.74 ± 0.81 as compared to 8.79 ± 1.33), but this did not reach statistical significance (p = 0.126) (Figure 1). Both resveratrol and EGCG had a significant influence on the latency of tumor development (Table 2 and Figure 2A, B, and 2C). Rats exposed to resveratrol had a significantly delayed onset of the first mammary tumor (76 days) as compared to the control animals (57 days; p < 0.05). Animals exposed to EGCG had a slight, but significantly enhanced onset of the first mammary tumor (51 days) compared to control rats (57 days; p < 0.05). This trend of delayed time to tumor development continued for the second and third times to tumor development in the resveratrol-treated rats, while the animals treated with EGCG were not significantly different than the control group (Table 2). Neither EGCG nor resveratrol had an effect on mammary tumor incidence as almost every animal (93 out of 94) developed at least one tumor by the end of the study. One resveratrol-treated animal did not develop any tumors. All histologically evaluated tumors were found to be adenocarcinomas. These results demonstrated that treatment of resveratrol in the diet significantly reduced the number and increased the time to onset of DMBA-induced mammary tumors. EGCG treatment showed no significant difference in the number

Table 1: Body weights and uterine to body weight ratios for female rats exposed to resveratrol or EGCG.

Treatment Age	Body Weight (g)	Uterine: BW Ratio
Control 21 Day	53.30 ± 1.09	0.57 ± 0.02
Resveratrol 21 Day	53.45 ± 1.25	0.56 ± 0.03
EGCG 21 Day	53.80 ± 0.67	0.53 ± 0.03
Control 50 Day	186.29 ± 3.25	1.79 ± 0.12
Resveratrol 50 Day	183.45 ± 2.84	1.95 ± 0.10
EGCG 50 Day	190.80 ± 2.63	1.54 ± 0.08

Female offspring of Sprague-Dawley CD rats were exposed to resveratrol (1 g/kg AIN-76A diet), EGCG (0.065% in drinking water), or AIN-76A alone as the control diet from birth to either 21 or 50 days postpartum. Values represent means ± SEM.

of mammary tumors per rat compared to the control animals.

Mammary gland differentiation, cell proliferation, and apoptosis

The predominant mammary terminal ductal structures found in 50-day-old control rats were terminal end buds (37%), terminal ducts (31%), lobules type I (23%), and lobules type II (9%). While resveratrol in the diet did not significantly alter the numbers of individual structures, the combined lobules types I and II were significantly increased compared to the control group (Table 3). On the other hand, EGCG in the diet significantly decreased the number of terminal ducts but did not affect terminal end buds, lobules I or lobules II.

BrdU incorporation was used as an index of cell proliferation. In the mammary glands of 50-day-old control rats, the labeling indices were ~5% in terminal end buds and terminal ducts and ~2% in lobules type I. Treatment with resveratrol in the diet significantly reduced cell proliferation in all of the terminal ductal structures that were measured (terminal ducts, terminal end buds, and lobules I) (Table 4). EGCG treatment did not alter the proliferation indices for any of the mammary terminal ductal structures as compared to the control group.

A DNA fragmentation assay was used to measure apoptosis. An apoptotic index was determined for mammary terminal end buds and lobules (combined type I and II) from 50-day-old rats. Rats treated with resveratrol showed a significant increase in apoptotic index in the mammary epithelial cells in terminal end buds as compared to the control rats (Table 5). EGCG in the diet did not have an effect on apoptosis in the epithelial cells of terminal end buds as compared to the controls. Neither treatment affected the apoptotic index in mammary lobules.

Serum polyphenol, 17β-estradiol, progesterone, and prolactin concentrations

Resveratrol and EGCG concentrations were measured in the serum of 50-day-old animals. In the serum of rats exposed to resveratrol in the diet, total resveratrol concentration was 2.00 ± 0.64 μM. In the serum of the EGCG treated group, at 50 days of age, the average concentration of total EGCG was 31.06 ± 3.05 nM. All rats on the control diet (AIN-76A) had non-measurable serum concentrations of resveratrol or EGCG.

The concentrations of 17β-estradiol, progesterone, and prolactin were measured in the serum of 50-day-old rats treated with resveratrol or EGCG in the diet or drinking water respectively (Table 6). There were no statistical differences in serum estradiol levels between control (11.39 ± 4.56 pg/ml), resveratrol-treated (13.87 ± 2.28 pg/ml), or

EGCG-treated rats (5.90 ± 1.64 pg/ml). Likewise, serum progesterone concentrations were not altered by resveratrol (14.98 ± 2.73 ng/ml) or EGCG treatment (9.64 ± 1.45 ng/ml) as compared to the control rats (12.42 ± 2.70 ng/ml). Neither resveratrol nor EGCG treatment affected serum prolactin concentrations as compared to the controls (1.24 ± 0.06, 1.19 ± 0.04, and 1.25 ± 0.10 respectively).

Discussion

Body and uterine weights and vaginal opening

Resveratrol and EGCG given throughout life at concentrations of 1 g resveratrol/kg diet and 65 mg EGCG/100 mL water, respectively did not cause any significant alterations on body or uterine weights in 21- and 50-day-old female Sprague-Dawley rats. Dietary treatment throughout life encompasses lactational exposure until time of weaning at day 21 postpartum, plus eating and drinking on their own after day 14. The number of days until vaginal opening, a marker for sexual maturation, was measured with no significant difference between any of the groups. Resveratrol and EGCG at the given concentrations did not cause toxicity as observed by loss of weight or differences in food and drink consumption. The lack of alteration of body weights was expected as Juan *et al.* showed that daily administration of resveratrol (20 mg/kg) had no effect on final body weights or on the tissue weights of the lungs, heart, liver, kidney, or adrenal glands [17]. Likewise, Hirose *et al.* showed no differences in body, liver, or kidney weights after treatment with green tea catechins in the diet or in the water [18].

Chemoprevention

Resveratrol given throughout life in the diet resulted in a suppression of DMBA-induced mammary cancer. Rats exposed to resveratrol had significantly fewer tumors per animal compared to those that were not exposed to resveratrol, and the latency (time to first, second and third tumors) was also significantly extended in the animals exposed to resveratrol. The effect of increasing tumor latency became more pronounced with each tumor. The chemoprotective effect of resveratrol on the mammary gland supports previous reports that resveratrol can suppress chemically-induced mammary carcinogenesis. Bhat *et al.* showed protection with resveratrol *via* gavage against NMU-induced mammary cancer, while Banerjee *et al.* showed protection against DMBA-induced mammary tumorigenesis [8,9], although the latter study was conducted with a small number of animals and used a suboptimal dose of DMBA which resulted in less than 80% tumor incidence in the control group and fewer than 2.5 tumors per rat. We chose to use a statistically more robust 30 animals per group and a dose of DMBA that resulted in 100% incidence of mammary adenocarcinomas in the control group over the course of the experiment, with an
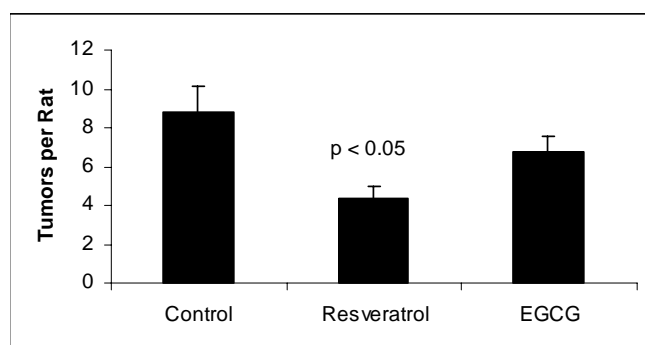
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Figure 1
Tumor multiplicity in female Sprague-Dawley CD rats exposed to resveratrol, EGCG, or AIN-76A (control) from birth until sacrifice. On day 50 postpartum, all animals were treated with 60 mg DMBA/kg body weight. Figure 1 shows tumor multiplicity, with the values representing mean tumors per rat ± 2 standard errors. A p value < 0.05 was considered statistically significant.

average of more than 8 tumors per rat in the control group. All tumors examined histologically were determined to be adenocarcinomas.

Exposure to 0.065% EGCG in the drinking water, throughout life, failed to protect the mammary gland against DMBA-induced mammary cancer. As mentioned above, the dose of EGCG was equivalent to one that inhibited prostate tumors in the TRAMP model with green tea polyphenol extract [12]. The tumor multiplicity in the EGCG-treated group was not significantly different from the animals that did not receive EGCG. While the time-to-

first tumor latency was slightly (yet significantly) shorter than that of the control animals, the times-to-second and -third tumors did not differ from that of the controls. We interrupt this to mean that pure EGCG, at the given dose, did not increase or decrease chemically-induced mammary cancer. Not finding a chemopreventive effect using EGCG is in line with several reports that show EGCG and green tea as having minimal effects on mammary carcinogenesis. Hirose *et al*. showed that a green tea polyphenol fraction had no effect on tumor incidence or multiplicity in a DMBA-induced mammary cancer model [18,19]. This lack of effect could be due to several factors, including the low reported bioavailability of EGCG [20]. Interestingly, Chen *et al*. reported that rats receiving decaffeinated green tea displayed a higher plasma concentration of EGCG than rats receiving pure EGCG, even though the dose of pure EGCG was five times higher [21]. This could implicate the other catechins found in green tea as playing a role in the bioavailability of EGCG and possibly the mammary-protective effects using whole green tea polyphenols or a mixture of green tea catechins [22,23]. It is important to study whether EGCG is the most active player in green tea or whether it is the mixture of catechins that is important for the health related effects.

Mammary gland differentiation
The effects of resveratrol and EGCG as given through the diet and drinking water, respectively were evident by the changes in the number and distribution of the types of mammary terminal ductal structures. At 50 days post-partum, the time of carcinogen exposure, there was no statistical difference in the numbers of terminal end buds between any of the groups. Terminal end buds are the

Table 2: Tumor latency to first, second and third tumor development.

Treatment	Median Time to First Tumor	p value
Control	57 days	-
Resveratrol	76 days	0.029
EGCG	51 days	0.017

Treatment	Median Time to Second Tumor	p value
Control	73.5 days	-
Resveratrol	99 days	0.014
EGCG	62 days	0.239

Treatment	Median Time to Third Tumor	p value
Control	81.5 days	-
Resveratrol	116.5 days	0.009
EGCG	85.5 days	0.309

Female offspring of Sprague-Dawley CD rats were exposed to resveratrol (1 g/kg AIN-76A diet), EGCG (0.065% in drinking water), or AIN-76A alone as the control diet starting at birth and continued throughout life. Each group contained a minimum of 30 rats. DMBA was administered at 60 mg/kg body weight at 50 days postpartum. The rats were palpated for tumors twice per week and the day of each tumor appearance was recorded. All p values are measured against the control group.
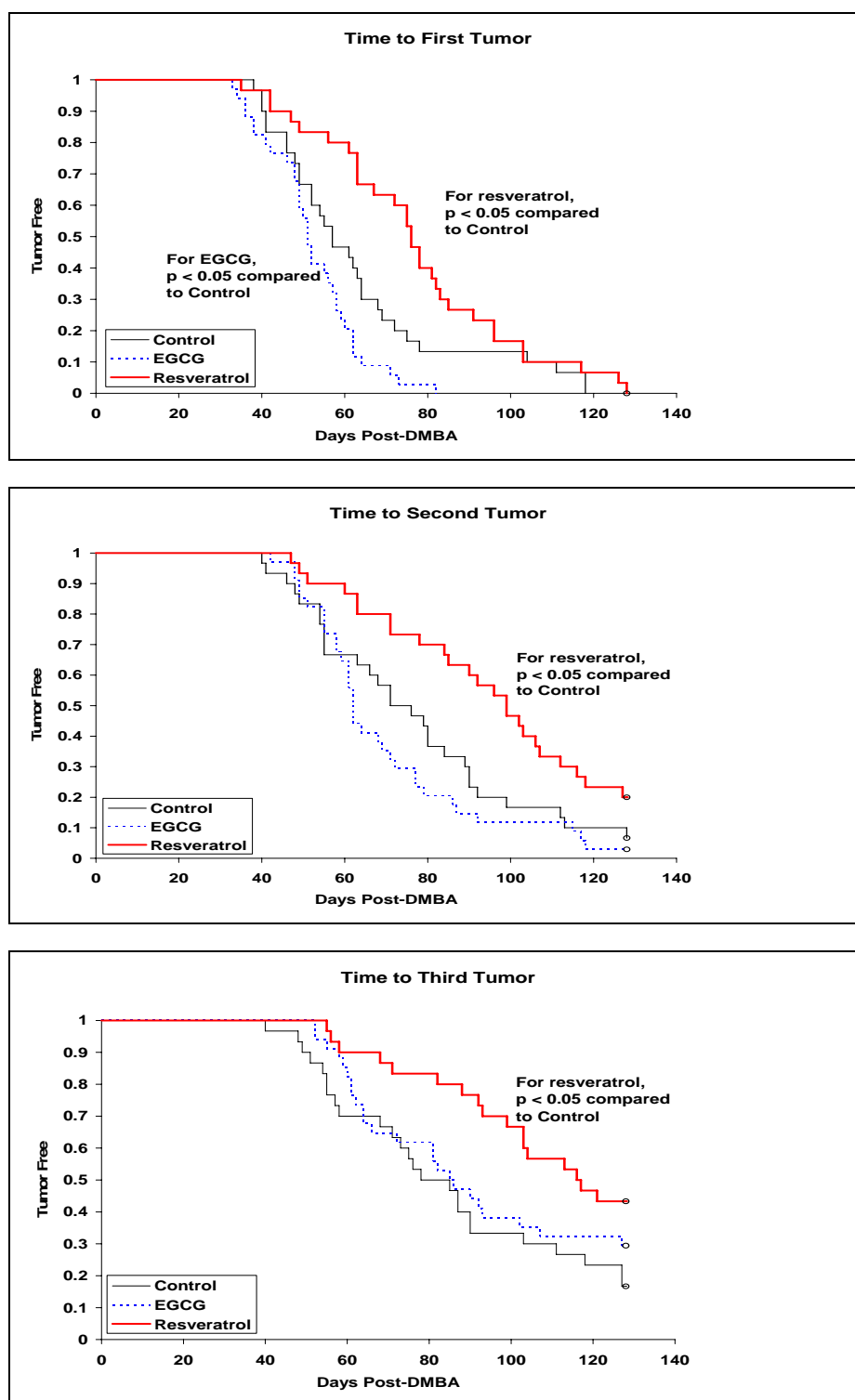
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Figure 2
Tumor latency (2A, B, C) in female Sprague-Dawley CD rats exposed to resveratrol, EGCG, or AIN-76A (control) from birth until sacrifice. On day 50 postpartum, all animals were treated with 60 mg DMBA/kg body weight. Figures 2A, 2B, and 2C depict the time to first tumor, second tumor, and third tumor (latency), respectively. A p value < 0.05 was considered statistically significant.

Table 3: Terminal ductal structures in mammary glands of female rats exposed to resveratrol or EGCG in the diet.

Treatment	Terminal Ducts	Terminal End Buds	Lobules Type I	Lobules Type II	Lobules Type I and II
Control	44 ± 5	53 ± 3	32 ± 3	13 ± 2	44 ± 4
Resveratrol	41 ± 6	58 ± 3	42 ± 3	15 ± 3	59 ± 5[A]
EGCG	28 ± 3[A]	39 ± 9	34 ± 4	16 ± 2	50 ± 4

Female offspring of Sprague-Dawley CD rats were exposed to resveratrol at 1 g/kg AIN-76A diet, 0.065% EGCG in the drinking water, or AIN-76A diet alone from birth to 50 days postpartum. The fourth abdominal mammary gland was measured in a minimum of seven rats per group. Values for terminal ductal structures represent means ± SEM. [A] represents a p value < 0.05. The p value is compared to the control group.

least differentiated terminal ductal structures in the mammary gland and reported to be the most susceptible to carcinogen exposure [13]. Reduction in the number of terminal end buds has been correlated with a reduction in tumor multiplicity in rats exposed prepubertally to the phytoestrogen genistein [2-4]. The animals exposed to resveratrol throughout life had slightly, though significantly, more combined lobule structures at 50 days post-partum. Lobules types I and II are the most differentiated structures and this increase in gland maturation can help to explain the protective effects seen with exposure to resveratrol *via* the diet. Chemoprevention experiments in our lab with the phytoestrogen genistein, a component of soy, have shown a reduction in mammary tumor multiplicity correlated with fewer terminal end buds and increased lobules at 50 days postpartum [2-4]. Hilakivi-Clarke *et al.* also showed that the mammary glands of rats treated with high n-3 polyunsaturated fatty acids have more lobules and are less susceptible to carcinogen insult [24]. Thus, enhanced maturation of the mammary gland can protect against carcinogen insult. EGCG-treated animals showed no increase in lobule structures and no decrease in the number of terminal end buds, which could influence the lack of protection against carcinogenesis.

Cell proliferation
Cellular proliferation in susceptible tissue structures is extremely important in the oncogenic-response to carcinogens. Russo *et al.* have shown a greater oncogenic response in active, dividing cells in the mammary gland of rats, with the active cells in the terminal end buds being the most responsible for subsequent, DMBA-induced adenocarcinomas [13,25-27]. In our work, at 50 days post-

partum, resveratrol-treated rats showed a significant reduction in the percentage of proliferating cells in the mammary terminal end buds as well as in terminal ducts and lobules. This reduction in proliferative cells in the terminal ductal structures of the mammary gland could contribute to the protective effect against mammary carcinogenesis. On the other hand, the labeling index for EGCG-treated rats in all terminal ductal structures was not statistically different than the control rats. No reduction of cell proliferation at 50 days postpartum could help to explain the lack of mammary cancer suppression observed with EGCG as compared to resveratrol.

Apoptosis
The apoptotic labeling index for epithelial cells in mammary terminal end buds in the resveratrol-treated animals was increased 25% compared to the control rats. Though a modest increase, the increase in apoptosis reached statistical significance. The rats treated with EGCG showed no statistical difference in apoptotic index in the mammary terminal end buds compared the control animals. Neither treatment affected the apoptotic index for mammary lobular structures. Resveratrol has been reported to induce apoptosis in a number of cancer cell lines, including breast carcinoma cell lines [reviewed in [6]]. This increase in mammary epithelial cell apoptosis in the terminal end buds after resveratrol treatment, coupled with the significant reduction in cell proliferation could create an environment in the mammary gland that is less susceptible to chemical carcinogenesis.

Table 4: Cell proliferation in mammary terminal ductal structures of 50-day-old rats exposed to resveratrol or EGCG.

Treatment	Terminal End Buds	Terminal Ducts	Lobules
Control	4.83 ± 0.61	5.03 ± 1.07	1.75 ± 0.22
Resveratrol	2.50 ± 0.75[A]	0.79 ± 0.27[A]	0.90 ± 0.14[A]
ECGC	4.56 ± 0.31	4.54 ± 0.97	2.15 ± 0.31

Female Sprague-Dawley CD rats were exposed from birth to 50 days postpartum to: resveratrol (1 g/kg diet), EGCG (0.065% in water), or AIN-76A as the control. Two hours prior to sacrifice the rats were injected with 100 mg BrdU/kg body weight. The labeling index was determined in three of each type of terminal structure (terminal ducts, terminal end buds, lobules) per mammary gland. A minimum of 7 rats was used in each group. Values represent means ± SEM. [A] represents a p value < 0.05.

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Table 5: Apoptotic index for epithelial cells in mammary terminal ductal structures of rats 50 days postpartum exposed to resveratrol or EGCG.

Treatment	Terminal End Buds	Lobules
Control	35.43 ± 1.37	39.39 ± 2.67
Resveratrol	44.18 ± 1.84[A]	41.24 ± 2.38
Control	46.10 ± 1.82	43.91 ± 1.97
EGCG	49.31 ± 1.57	43.80 ± 2.52

Female Sprague-Dawley rats were exposed to resveratrol or EGCG in the diet from birth until 50 days postpartum. Apoptotic indices (positively stained cells/total cells) were determined from mammary epithelial cells from terminal end buds and lobule structures, with at least 7 animals per group. Values represent mean ± SEM. [A] represents a p value < 0.05.

Serum polyphenol concentrations

Bioavailability is an important consideration when dealing with chemopreventive agents that are consumed in the diet. There are multiple reports that have measured the serum polyphenol concentrations in humans and rodents of both resveratrol and EGCG. At the present dose of resveratrol given in the diet, 1 g/kg diet, a 200 gram rat that consumed 20 grams of diet would receive ~100 mg resveratrol/kg body weight. The total resveratrol concentration measured in the serum of these resveratrol treated rats was 2.00 ± 0.64 µM. Crowell *et al*. administered 300 mg resveratrol/kg body weight/day by gavage and reported a serum concentration of 1.46 µM [28]. Resveratrol treatment, administered via gavage to rats at 20 and 50 mg/kg body weight respectively [29,30], gave maximum serum concentrations in the low micromolar range, consistent with our results. Our results were also similar to the total serum concentrations of resveratrol in humans (12 healthy males) receiving 0.36 mg resveratrol/kg body weight dissolved in white wine [31]. This dose is about 20 times higher than the amount of resveratrol one could receive after moderate wine consumption. Interestingly, Gescher and Steward point out in a commentary that low doses of resveratrol, and thus low resveratrol serum concentrations, may suffice to exert a potent chemopreventive effect [32]. Further studies are necessary to discern the effects of lower doses of resveratrol on mammary carcinogenesis.

In our study with EGCG, female rats received 0.065% EGCG in the drinking water, made fresh each afternoon. Thus, a 200 g female consuming 25 mL of fluid would receive 16.25 mg EGCG per day (81 mg EGCG/kg body weight). Again, this dose was extrapolated from the work of Gupta *et al*. who showed that a 0.1% enriched fraction of green tea polyphenols (62% EGCG) could inhibit prostate cancer in the TRAMP mouse model [12]. That report also stated that the dose "mimics an approximate consumption of six cups of green tea per day by an average adult human." At the dose administered, the total EGCG concentration found in the serum was 31.06 ± 3.05 nM. This is similar to the work of Chen *et al*. who treated male rats with 75 mg EGCG/kg body weight by gavage and reported a serum concentration of total EGCG at 43 nM [21]. Several human studies have employed doses of EGCG that would be equivalent to ~2–3 cups of green tea for an average human. Lee *et al*. administered 20 mg green tea solids/kg body weight and reported a maximum serum EGCG concentration of 172 nM [33]. Another group using a similar dose reported a maximum serum concentration of total EGCG at ~720 nM [34]. Species differences could account for the lower serum concentrations of EGCG observed in the rat. It has been reported that most of the EGCG does not get into the blood, but is excreted through the bile to the colon [21]. Also, higher serum concentrations of EGCG have been reported when green tea polyphenol mixtures have been administered as opposed to pure EGCG [21]. The low circulating polyphenol concentration of EGCG could play a role in the lack of effect observed on tumor multiplicity and latency observed in this study.

Table 6: Serum concentrations of 17β-estradiol, progesterone, and prolactin in 50-day-old female rats, exposed to resveratrol or EGCG.

Treatment	17β-Estradiol (pg/ml)	Progesterone (ng/ml)	Prolactin (ng/ml)
Control	11.39 ± 4.56	12.42 ± 2.70	1.25 ± 0.10
Resveratrol	13.87 ± 2.28	14.98 ± 2.73	1.19 ± 0.04
EGCG	5.90 ± 1.64	9.64 ± 1.45	1.24 ± 0.06

Female Sprague-Dawley CD rats were exposed from birth to 50 days postpartum to: resveratrol (1 g/kg diet), EGCG (0.065% in water), or AIN-76A as the control. Blood was collected from female rats determined to be in the estrous phase of the estrous cycle (8/group). 17β-estradiol, progesterone, and prolactin concentrations were measured by radioimmunoassay. Values represent the mean ± SEM.

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Serum 17β-estradiol, progesterone, and prolactin concentrations

At 50 days postpartum, resveratrol in the diet or EGCG in the drinking water did not significantly influence the serum concentrations of estradiol, progesterone, or prolactin. The serum concentrations of estradiol for control, resveratrol-, and EGCG-treated animals were 11.4, 13.9, and 5.9 pg/ml, respectively. Lamartiniere et al. have reported similar serum estradiol concentrations (~10–12 pg/ml) after dietary treatment with AIN-76A or 250 mg daidzein/kg diet [35]. The same report showed serum progesterone concentrations in the low ng/ml range, very similar to what we saw with serum progesterone concentrations after resveratrol or EGCG treatment. Though not reaching statistical significance, EGCG tended to lower serum estradiol concentrations as compared to the control rats (5.90 ± 1.64 versus 11.39 ± 4.56 nM). This is in line with the previous reports of Kao *et al.* [36] and Hangata *et al.* [37] who showed in rats and humans, respectively that green tea treatment could lower serum estradiol concentrations. Serum prolactin was not significantly altered by treatment with resveratrol or EGCG. This is consistent with Okazaki *et al.*, who reported no difference in serum prolactin levels after exposure to the polyphenol genistein [38].

Summary

Our work supports the previous reports that resveratrol in the diet is effective at inhibiting DMBA-induced mammary cancer. We have shown that resveratrol can enhance maturation of the mammary gland as well as reduce cellular proliferation and increase apoptosis in mammary epithelial cells, in a manner that is protective against mammary carcinogenesis. Coupling this with our previous work with genistein [2-4], we plan to use a combination of genistein and resveratrol in the diet to determine whether there is an additive or synergistic effect of these natural polyphenols to suppress mammary carcinogenesis. As for the administration of EGCG in the drinking water, no mammary protective effect was demonstrated. It remains to be seen whether a higher dose, different route of administration, a different tea catechin, or a mixture of green tea catechins can protect against mammary cancer.

There is still much that remains unknown about the *in vivo* molecular mechanisms of resveratrol and EGCG that play a role in their effects on mammary carcinogenesis. The effects on estrogen receptor pathways as well as other steroid and growth factor pathways and effects on enzymes that are important in the activation or removal of xenobiotics must be elucidated such that these polyphenols can be recommended for clinical trials and help in the prevention of breast cancer.

Authors' contributions

TGW carried out the carcinogenesis study, whole mount analysis, cell proliferation and apoptosis assays, and drafted the manuscript. MC performed the statistical analysis for tumor multiplicity and tumor latency, wrote the statistical methods, and assisted in writing the manuscript. CAL proposed the study design and assisted in writing the manuscript.

Acknowledgements

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APPENDIX C.

A pdf version of the published review entitled: "Genistein and resveratrol: mammary cancer chemoprevention and mechanisms of action in the rat."

Genistein and resveratrol: mammary cancer chemoprevention and mechanisms of action in the rat

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The environment, including diet, plays a critical role in a woman's subsequent risk of breast cancer. Two dietary polyphenols that have received attention from the health and research communities for their ability to protect against breast cancer are: genistein, a component of soy; and resveratrol, a phytoalexin found in red grapes and red wine. We and others have shown that both genistein and resveratrol can protect against mammary cancer in rodents. The timing of exposure to genistein appears critical for its mammary protective effects. It has been reported that genistein early in life causes enhanced mammary gland differentiation, alterations in cell proliferation and apoptosis, and upregulation of tumor-suppressor genes. With resveratrol in the diet, changes in cell proliferation and apoptosis in terminal ductal structures of the mammary gland might help to explain its protective effects. We conclude that genistein and resveratrol can protect against breast cancer by regulating important mammary growth and differentiation pathways.

Breast cancer remains a destructive disease throughout the world. In the USA alone, there are expected to be almost 213,000 new cases of breast cancer and 41,000 deaths this year [1]. Common genetic mutations, such as *BRCA-1* and *BRCA-2* mutations, account for only a small percentage of the overall incidence of the disease. It is curently believed that certain lifestyle factors can reduce a woman's risk of breast cancer [2]. Asian countries, such as China, have a much lower incidence of breast cancer compared with western nations. Some investigators have proposed that the typical Asian diet, high in soy foods, may help to explain the lower breast cancer incidence in these countries [3–4]. Genistein is the major isoflavone that is found in soy. The importance of this lifestyle (diet) is clearly high as it has been shown that immigration to the USA results in a loss of breast cancer protection by the second generation [5]. Resveratrol, a component of red grapes and red wine, has also received a lot of attention for its beneficial health effects, especially against cardiovascular disease [6]. Recently, *in vitro* and

in vivo studies suggested that resveratrol may be effective against certain cancers, including breast cancer.

Cancer has long been considered a disease of the aging population. It is often not treated until diagnosis, a point at which the cancer may be advanced. It has become increasingly clear that exposures early in life can play a major role in cancer development [2]. Regarding women and breast cancer, ionizing radiation and pregnancy illustrate the importance of early life exposures. Women exposed as teenagers to ionizing radiation are more susceptible to breast cancer than those exposed as adults [7,8]. By contrast, early pregnancy or early exposure to the hormones of pregnancy reduces the incidence of breast/mammary cancer in women and animal models [9–12]. Thus, the timing of certain chemical/hormonal exposures is critical for the effect on later breast cancer risk.

Our laboratory has focused on the prevention of breast cancer using natural polyphenols, such as genistein and resveratrol. Many of the polyphenols have activities as estrogen agonists or antagonists, regulators of

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growth factor signaling pathways and modulators of cell differentiation. We have hypothesized that exposure to these polyphenols early in life will institute a developmental maturation that could result in a mammary gland that is less susceptible to cancer later in life. These polyphenols can modulate mammary gland architecture and differentiation, mammary epithelial cell proliferation and apoptosis, and other critical sex steroid and growth factor pathways that play a role in both the normal and cancerous development of the mammary gland.

Genistein: a soy isoflavone

Genistein has been extensively studied over the last decade. Genistein (4,5,7-trihydroxyisoflavone) is a naturally occurring isoflavone and the major metabolite of soy (FIGURE 1). It was originally identified as possessing weak estrogenic activity and has a chemical structure that resembles estradiol. It is now known that genistein binds to the estrogen receptors α and β, with a higher affinity for the latter [13]. The biochemical mechanisms of genistein and the inhibition of carcinogenesis *in vitro* have been thoroughly reviewed in the literature [14-16]. Genistein is reported to be an *in vitro* inhibitor of protein tyrosine kinases, topoisomerases I and II, and 5α-reductase. The inhibition of tyrosine kinases may help to explain the inhibition of growth in many cancer cell lines, including breast and prostate. Genistein can also induce cell-cycle arrest and apoptosis in cancer cell lines, as well as inhibit the activation of transcription factors, such as nuclear factor (NF)-κB, and known growth-stimulating pathways, such as the Akt and MAP kinase pathways. Drawbacks to these *in vitro* studies have been the use of high polyphenol concentrations (~50 μM) and conflicting data with the use of low and high concentrations of genistein. The plasma level of genistein in people on a soy-rich diet was reported to be approximately

Figure 1. Estradiol, genistein and resveratrol.

276 nM [17]. Differences derived from *in vitro* experiments create the need for *in vivo* experimentation with nutritionally relevant doses of soy and genistein.

Genistein & mammary cancer: *in vivo* chemoprevention

In 1995, our laboratory reported that genistein, the primary isoflavone component of soybeans, administered via subcutaneous injections to Sprague–Dawley rats, suppressed dimethylbenz[a]anthracene (DMBA)-induced mammary tumorigenesis [18]. Later, using a physiologically relevant protocol, we demonstrated that genistein in the diet was also protective against DMBA-induced mammary cancer [19]. In these experiments, rats were exposed to increasing concentrations of genistein in the diet from conception to 21 days postpartum. The offspring were then treated with 80 mg DMBA/kg body weight at 50 days postpartum to induce mammary cancer. With dietary concentrations of genistein 0, 25 and 250 mg/kg AIN-76A diet (phytoestrogen free), there was a dose-dependent protection against mammary tumor multiplicity, as measured by the number of tumors per rat. It should be noted that the genistein 250 mg/kg diet resulted in a serum genistein concentration (726 nM) comparable with those found in Asian men eating a traditional diet high in soy [17].

Several other exposure periods of genistein have been tested for mammary cancer protection. *In utero*-only administration of genistein 250 mg/kg diet failed to protect the female offspring against chemically induced cancer, although it did not enhance DMBA-induced mammary tumorigenesis (TABLE 1) [20,21]. Our laboratory also tested the effects of genistein on tumor promotion. Female rats were gavaged with DMBA at 50 days postpartum. Starting at 7 weeks after exposure to the carcinogen (approximately the time to first palpable tumor), animals were fed a genistein 250 mg/kg diet. There were no observed differences in tumor formation or adenocarcinoma development compared with animals that did not receive genistein. This demonstrates that genistein ingested after the start of tumor development does not promote mammary cancer. Another experiment investigated exposure to genistein in the diet, both prepubertally and as an adult. Rats that received genistein at both time points were protected further than those that only received genistein before puberty (TABLE 1). We concluded that early prepubertal exposure to genistein is the critical time for the initial mammary protective effects. These dietary chemoprotective effects of genistein have been confirmed by other laboratories [22-24].

A few studies have reported no effect or a stimulating effect of genistein on mammary tumorigenesis [25]. Most of these studies investigated adult exposures or exposures after the induction of tumorigenesis to soy or genistein. However, they did not treat the animals prepubertally with genistein, an important aspect of this novel chemoprevention. Studies have also been carried out in ovariectomized mice that were immunocompromised and implanted with transformed human breast cancer cells. Administering genistein to this model resulted in a promotional effect on cancer cell growth [26-29]. On the other hand, in another mouse model of
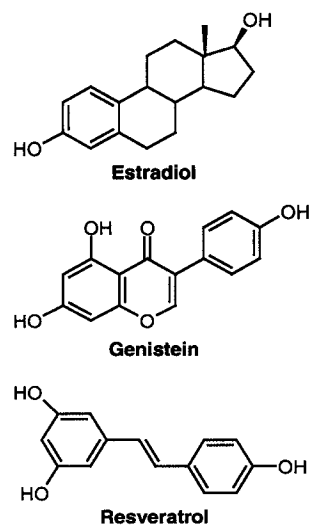
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Table 1. Genistein timing of exposure and mammary cancer chemoprevention.

Exposure period	Relative mammary tumor multiplicity
No genistein	8.9
Prenatal (*in utero*) genistein (throughout gestation)	8.8
Adult genistein (starting at 100 days postpartum)	8.2
Prepubertal genistein (days 1–21 postpartum)	4.3
Prepubertal + adult genistein (days 1–21 and 100+)	2.8

All exposures to genistein were at 250 mg/kg AIN-76A diet. At 50 days postpartum, all female offspring were treated with dimethylbenz[a]anthracene (80 mg/kg of body weight) to induce mammary tumorigenesis.
Adapted from [21] with permission from the American Society for Nutrition.

breast cancer in which the ovary was left intact, genistein prevented the appearance of mammary tumors [30] or increased the latency period before tumors appeared [31]. These studies highlight the importance of the model system and timing of exposure to soy or genistein.

An important consideration of any cancer causation and prevention study is how the animal studies reflect the human situation. Following our reports of prepubertal genistein suppressing mammary tumor development in rats, epidemiology studies were conducted and the results support the concept of timing of exposure to genistein providing a protective effect. Recent epidemiology reports showed the importance of the adolescent period, demonstrating a significant reduction of breast cancer risk with adolescent (13–15 years old) consumption of soy [32].

Genistein mechanisms of action

Our laboratory and others have clearly demonstrated that genistein administered prepubertally in the diet can protect against chemically induced mammary cancer. We have investigated mammary gland maturation as a potential mechanism for chemoprevention. Rats treated prepubertally with genistein in the diet were sacrificed at 50 days postpartum (time of carcinogen administration in the tumorigenesis studies) to observe changes in mammary maturation and architecture. At 50 days postpartum, there was a significant decrease in the number of terminal end buds (TEBs) present in mammary gland whole mounts, consistent with a more mature mammary gland [19]. Russo and colleagues have previously demonstrated that mammary TEBs have highly proliferative cells that are the most susceptible to carcinogenic insult, while the more mature lobules are less susceptible terminal ductal structures [33]. Thus, with dietary genistein treatment there are fewer structures that may be susceptible to a carcinogen, such as DMBA. This is akin to an explanation by which early pregnancy may protect a woman against breast cancer later in life [34]. The maturation associated with

pregnancy and lactation results in a gland that is biologically mature (with a different genomic signature and different ability to respond to insult) than that of a nulliparous woman. This maturation and reduction in TEBs was consistent with the effects of pharmacological injections of genistein. Neonatal or prepubertal injections of genistein resulted in a decrease in the number of TEBs and an increase in lobular structures at 50 days postpartum [20,21]. Cell proliferation was also less in the mammary terminal ductal structures and there was a significant decrease in the protein level of the epidermal growth factor receptor (EGFR), which could help to explain the decrease in cell proliferation [35]. Increased apoptosis in the mammary glands of rats exposed to genistein early in life has been reported with a significant increase in the tumor-suppressor protein PTEN (phosphatase and tensin homolog deleted in chromosome 10) [36]. Cabanes and colleagues demonstrated that prepubertal exposure to genistein or estradiol (both via subcutaneous injection) upregulated the mRNA expression of BRCA1, a known tumor-suppressor gene [37]. Thus, prepubertal exposure to genistein may protect against mammary cancer by a host of mechanisms including: enhanced mammary maturation, decreased cell proliferation, increased apoptosis and increased expression of tumor suppressors, such as PTEN and BRCA1.

More recently, our laboratory has used proteomic technologies to investigate novel mechanisms of genistein chemoprevention [38]. Rats were treated with or without genistein at days 16, 18 and 20 postpartum. At 21 days postpartum, mammary glands were subjected to 2D polyacrylamide gel electrophoresis. This discovery technique allowed a system-wide resolution of proteins that are differentially expressed between groups. Six proteins were found to be differentially expressed, with one, GTP-cyclohydrolase 1 (GTP-CH1) confirmed by immunoblot analysis. Further investigation of the downstream pathway of GTP-CH1 revealed that tyrosine hydroxylase was upregulated and vascular endothelial growth factor receptor (VEGFR)2 was downregulated in the mammary glands of 50-day-old rats treated with genistein in the prepubertal period. This result was consistent with, and could help to explain, the mammary gland maturation and cell proliferation data that had been observed previously. This report demonstrated the importance of using new 'systems biology' approaches to look for novel mechanisms of action after treatment with phytochemicals.

Resveratrol: more than just an antioxidant

Resveratrol (trans-3,4',5-trihydroxystilbene) (FIGURE 1), a phytoalexin found in grape skins and red wine, has received a lot of attention for its health-beneficial properties. Epidemiology reports have shown an inverse correlation between wine intake and death resulting from cardiac disease in French populations, an area with a high consumption of red meat, leading to what is known as the 'French Paradox' [6]. A more recent report demonstrated a significant inverse association between resveratrol from grape consumption and breast cancer risk [39]. These reports have coincided with numerous *in vitro* studies demonstrating the anticancer potential of resveratrol. Reviews
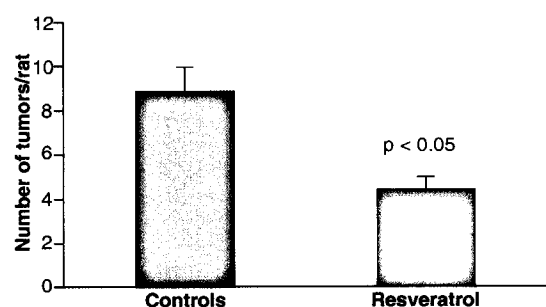
Figure 2. Resveratrol chemoprevention in a chemically induced rat model of mammary cancer. Animals were exposed to resveratrol 1 g/kg AIN-76A diet or AIN-76A diet only starting at birth. On day 50 postpartum, all rats were treated with dimethylbenz[a]anthracene 60 mg/kg body weight. Values represent the mean number of tumors per rat ± 2 standard errors. A p-value of less than 0.05 was considered statistically significant. Modified from [46].

by Bhat and Pezzuto, and Le Corre and colleagues summarize the numerous ways in which resveratrol might prevent breast and other cancers [40,41]. Many of these studies have investigated the antioxidant effects, modulation of cytochromes P450, effects on cell cycle and apoptosis, modulation of NF-κB, estrogenic/anti-estrogenic properties and effects on the phosphatidylinositol 3-kinase (PI3K) signaling pathway as possible explanations for the cancer chemoprevention. Resveratrol administration in these experiments showed no toxicity, even at supraphysiological concentrations. Again, these *in vitro* studies often use high, micromolar concentrations that may not be nutritionally relevant and creates a need for rodent and human studies with lower concentrations.

Resveratrol & mammary cancer chemoprevention

Jang and colleagues reported the chemopreventive properties of resveratrol against the initiation, promotion and progression of carcinogenesis *in vitro* [42]. In addition, there have been reports in which resveratrol has been shown to reduce the induction of chemically induced breast cancer models. Bhat and colleagues administered resveratrol via gavage and demonstrated an increase in tumor latency and a decrease in the total number of tumors in an N-methyl-N-nitrosourea (NMU)-induced mammary cancer model [43]. Dietary resveratrol also reduced incidence and multiplicity and extended the latency period in a DMBA-induced mammary cancer model, although this study used a small number of animals [44]. It should be noted that the study by Banerjee and colleagues showed chemoprevention using a very low dose of

resveratrol (100 mg/rat). In 2005, it was also reported that resveratrol treatment could delay the development of spontaneous mammary tumors in a HER-2/neu transgenic mouse model [45]. Tumor size and the number of lung metastases were also diminished by resveratrol treatment.

Our laboratory has recently shown that resveratrol administered via the diet from birth until the end of the study at 180 days can suppress DMBA-induced mammary tumors [46]. The administration of resveratrol in the diet resulted in a significant decrease in mammary tumor multiplicity (FIGURE 2) and a significant increase in tumor latency (i.e., time to first palpable tumor) (FIGURE 3). The treatment with resveratrol in the diet did not cause any toxicity as measured by body weight, food and drink consumption, and days to vaginal opening (a marker of sexual maturity). Serum concentrations of resveratrol in the female rats were 2.0 μM.

Resveratrol, mammary gland maturation, cell proliferation & pathway modulation

The mechanisms through which resveratrol exerts a cancer-protective effect on the *in vivo* mammary gland are just beginning to be elucidated. Our laboratory has shown that treatment with resveratrol in the diet can increase the number of lobular structures (lobule types I and II) in the mammary gland at 50 days postpartum [46]. This increase in gland maturation may help to explain the chemopreventive activity. We also showed a significant decrease in cell proliferation and increase in apoptosis in the TEBs with resveratrol treatment. Treatment with resveratrol may cause changes in the gland, making it less susceptible to carcinogenic insult. Other *in vivo*
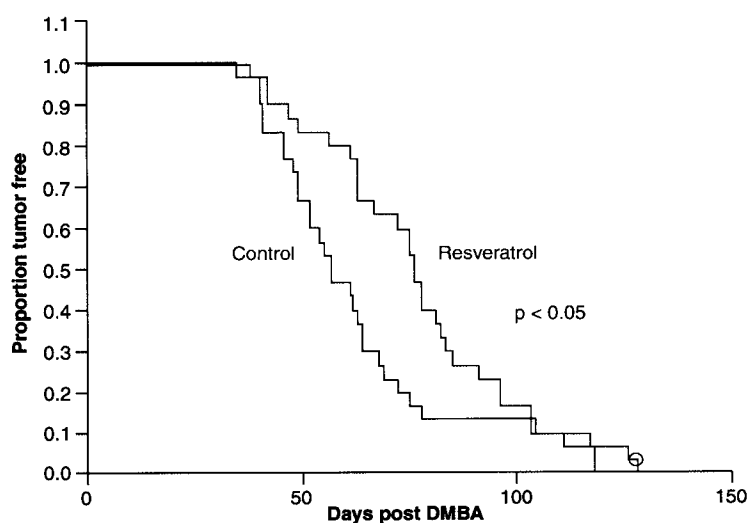

Figure 3. Resveratrol influence on mammary tumor latency. Animals were exposed to resveratrol 1 g /kg AIN-76A diet or AIN-76A diet only starting at birth. On day 50 postpartum, all rats were treated with 60 mg DMBA/kg body weight. Kaplan–Meier estimates of tumor-free survival were plotted. A p-value of less than 0.05 was considered statistically significant. DMBA: Dimethylbenz[a]anthracene. Figure modified from [46].

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work on the chemopreventive properties of resveratrol has implicated several molecules and pathways of known relevance to breast cancer. One report showed that resveratrol treatment could inhibit the DMBA-induced expression of cyclooxygenase-2 and matrix metalloprotease-9 expression in breast tumors [44]. NF-κB induction by DMBA was also suppressed by resveratrol. Resveratrol treatment of a HER-2/neu mammary cancer model demonstrated that *HER-2/neu* gene expression could be inhibited [45]. There is still much to be learned regarding the mechanisms through which resveratrol is able to protect the mammary gland from cancer.

Combination treatments of genistein & resveratrol

The combining of treatments in breast cancer for a more efficacious outcome is not a new idea. This strategy is already employed in the therapeutic fight against breast cancer, with multiple chemotherapy drugs that act by different mechanisms administered to better fight cancer and prevent toxicity. Recently, our laboratory has used combinational treatments of genistein and resveratrol, both of which have been shown to prevent breast cancer. This strategy has been effective using soy and green tea extract, as green tea and soy phytochemicals reduced tumor weight in an *in vivo* mammary cancer model beyond the reduction seen with green tea only [47]. We administered lower doses of genistein and resveratrol (83 and 333 mg/kg diet, respectively) alone and in combination (genistein 83 mg plus resveratrol 100 mg or resveratrol 333 mg plus genistein 25 mg/kg diet) from birth throughout life. Both genistein and resveratrol at the lower doses still protected the mammary gland against DMBA-induced tumors with a reduction in multiplicity and an increase in tumor latency (TABLE 2). When the genistein 25 mg/kg diet was added to the resveratrol 333 mg/kg diet treatment, a further reduction in tumor multiplicity was observed, although this did not reach statistical significance. Again, we noticed a trend toward an increased maturation of the mammary gland at 50 days postpartum and saw significant reductions in epithelial cell proliferation in the TEBs with the lower doses of genistein

and resveratrol. Combining mammary chemoprotective agents may improve the ability to suppress tumorigenesis by targeting different pathways, allowing lower doses to be effective and reducing the possibilities of toxicity.

Expert commentary

Our work clearly demonstrates the possibility and opportunity to beneficially affect breast cancer risk early in life and with dietary choices. The data from our and other labs on bioactive phytochemicals support epidemiological data showing that lifestyle and diet can influence the incidence of certain diseases, including breast cancer. We and others have shown that prepubertal genistein exposure in the diet can suppress chemically induced mammary cancer in rats. It has become evident that the timing of exposure is critical for the anticancer effects. We have demonstrated that genistein enhances mammary gland maturation with early life exposure, resulting in a gland that is less susceptible to cancer. We have also shown that resveratrol can suppress DMBA-induced mammary cancer with no apparent toxicity. Resveratrol treatment significantly decreased cell proliferation in the epithelial cells of mammary TEBs, the most susceptible structure for tumorigenesis. There remains much to elucidate regarding the molecular mechanisms by which these polyphenols exert chemoprevention so that these bioactive compounds can be recommended for clinical trials and aid in the prevention of breast cancer.

Five-year view

The field of cancer chemoprevention has become a major topic of interest and investigation in the medical and research communities. The use of natural products such as soy (genistein), red grapes/red wine (resveratrol) and many others is very appealing owing to the abundance and limited toxicity of these natural products. Genistein and resveratrol have clearly been shown to protect against both chemically induced and spontaneous mammary tumorigenesis in rodent models. The mechanisms of these polyphenols must now be elucidated.

Table 2. Mammary tumor multiplicity and latency in a chemically induced rat model of mammary cancer exposed to genistein, resveratrol or combinations of genistein and resveratrol in the diet.

	Tumors per rat (multiplicity)	% reduction compared with controls	p-value compared with controls	Median time to first tumor (latency)	p-value compared with controls
Controls	8.27	–	–	44 days	–
Genistein 83 mg/kg diet	3.43	58	<0.0001	63.5 days	<0.0001
Resveratrol 333 mg/kg diet	6.31	24	0.034	51 days	0.008
Resveratrol 333 mg/kg + genistein 25 mg/kg diet	5.29	36	<0.001	52 days	0.006
Resveratrol 100 mg/kg + genistein 83 mg/kg diet	5.50	33	<0.01	49.5 days	0.0024

Female Sprague–Dawley rats were exposed from birth throughout life to genistein, resveratrol, combinations of genistein and resveratrol, or control (AIN-76A) in the diet. All rats were exposed to dimethylbenz[a]anthracene (60 mg/kg body weight) at 50 days postpartum to induce tumorigenesis. A p-value of less than 0.05 was considered statistically significant.

The use of genomics and proteomics, techniques that investigate thousands of genes or proteins simultaneously, will be very useful for elucidating both known and novel pathways that are modulated by genistein and resveratrol. Another trend that is arising in the field of cancer chemoprevention is the use of combinational prevention. By elucidating the mechanism through which these polyphenols work, compounds that affect distinct pathways could be used in combination to further reduce breast cancer risks. This same strategy could help to reduce toxicity since lower doses of multiple compounds could be as or more effective than any single

exposure. There is much room for growth in this area, a field that could substantially affect the risk for developing what is remains a devastating disease.

Acknowledgement

Key issues

- Breast cancer remains a destructive disease despite the advancement of screening and therapeutics, and it has become increasingly clear that the environment (diet) and early life exposures play a critical role in determining a woman's risk for breast cancer.

- Genistein, a naturally occurring soy isoflavone, and resveratrol, a phytoalexin found in grape skins and red wine, have received a lot of attention for the health-beneficial properties.

- Genistein, administered early in life, can protect against chemically induced mammary cancer by enhancing gland maturation.

- Epidemiology reports confirm a decreased risk of breast cancer with adolescent consumption of soy and have also shown an inverse correlation between red grape intake and breast cancer risk.

- Our lab and others have shown that resveratrol can suppress mammary tumor multiplicity and increase tumor latency in a dimethylbenz[a]anthracene-induced rat model.

- Modulation of cell proliferation and apoptosis in critical mammary structures can help to explain the protection that is observed with resveratrol exposure.

- Techniques that look at entire 'systems' of genes and proteins may play a critical role in elucidating novel pathways that are important for the mechanisms of breast cancer causation and prevention.

- Our laboratory and others have demonstrated the potential of chemoprevention by combining polyphenols, such as genistein and resveratrol, which could decrease the chance of toxicity by decreasing the dose necessary for a protective effect.

- Our work and others' clearly demonstrates the possibility and opportunity to beneficially affect breast cancer risk early in life with lifestyle (dietary) choices.

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and tea bioactive components in mice.
Int. J. Cancer 108, 8–14 (2004).

• **Novel example of combining
chemopreventive agents to
enhance protection.**

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APPENDIX D.

The full abstract from each conference attended using data generated during the Pre-doctoral Training Award. Note that the meeting, date and location appear before the full abstract with authors.

1. Third Annual Early Environmental Exposures Meeting, Breast Cancer and Environment Research Centers. San Francisco, CA. Nov. 2006.

Mammary Cancer Chemoprevention with the Polyphenols Genistein and Resveratrol

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Despite recent advances in therapeutic treatments, breast cancer remains a devastating disease and a leading killer among female cancers. There is, and should be, a strong effort to work toward the prevention of this disease. It is well accepted that environmental factors, especially diet and lifestyle, play critical roles in determining one's risk for breast cancer. Two dietary polyphenols that have received much attention for their health benefits, including anti-cancer properties, are the soy isoflavone genistein and resveratrol, a phytoalexin found in red grapes and red wine. We have hypothesized that these two polyphenols given alone or in combination could suppress mammary cancer. Initially, we determined that genistein and resveratrol given from birth onward at 250 mg/kg and 1000 mg/kg AIN-76A diet, respectively can suppress dimethylbenz(a)anthracene (60 mg DMBA/kg body weight at day 50)- induced mammary carcinogenesis in female Sprague Dawley rats. Next, we treated rats from birth throughout life with one of five treatments: 1) Control (AIN-76A diet only), 2) 333 mg resveratrol/kg diet, 3) 83 mg genistein/kg diet, 4) 333 mg resveratrol + 25 mg genistein/kg diet, or 5) 100 mg resveratrol + 83 mg genistein/kg diet (30/group). A significant reduction in mammary tumor multiplicity and a significant increase in tumor latency were observed in all four polyphenol treatments. Interestingly, adding a low dose (25 mg/kg diet) of genistein to the resveratrol treatment, we observed a further reduction in tumor multiplicity. As to the mechanisms of chemoprevention, analysis of mammary whole mounts revealed that genistein enhanced gland differentiation and reduced cell proliferation. Resveratrol significantly decreased epithelial cell proliferation and increased apoptosis in mammary terminal ductal structures, the most susceptible mammary structure to carcinogenesis. Microarray analysis at 50 days postpartum revealed 386 and 470 genes that were differentially regulated between the resveratrol- and genistein-treated rats (respectively) versus control rats at a p value ≤ 0.01. Many of these genes provide possible mechanisms to our findings on altered mammary gland differentiation, cell proliferation, apoptosis, and a reduction in carcinogenesis. Resveratrol treatment modulated many survival and apoptosis-related genes including Akt, several cyclin-dependant kinases, and Bad. Genistein in the diet

affected several growth factor receptors including growth hormone receptor, IGF-2 receptor, and PPAR delta and other genes such as p53 and Bcl-2. The polyphenols genistein and resveratrol, alone and in combination, suppressed mammary tumorigenesis by modulating mammary gland differentiation and epithelial cell proliferation/apoptosis. More elucidation of these mechanisms should allow the use of these polyphenols in clinical trials and to prevent breast cancer. (Supported by NIH-NCI-P20-CA93753, DAMD17-00-1-0119, and DOD-BC043793)

2. Gordon Research Conference: Mammary Gland Biology. Lucca, Italy. June 2006.

Mammary Cancer Chemoprevention with the Polyphenols Genistein and Resveratrol

Timothy Whitsett[1], Dungtsa Chen[2,3], and Coral A. Lamartiniere[1,2],
[1]Department of Pharmacology and Toxicology, University of Alabama at Birmingham
[2]UAB Comprehensive Cancer Center, [3]Biostatistics and Bioinformatics Unit

Despite recent advances in therapeutic treatments, breast cancer remains a serious disease and a leading killer among female cancers. There is, and should be, a strong effort to work toward the prevention of this disease. It is well accepted that environmental factors, especially diet and lifestyle, play critical roles in determining one's risk for breast cancer. Two dietary polyphenols that have received much attention for their health benefits, including anti-cancer properties, are the soy isoflavone genistein and resveratrol, a phytoalexin found in red grapes and red wine. We have hypothesized that these two polyphenols given alone or in combination could suppress mammary cancer. Initially, we determined that genistein and resveratrol given from birth onward at 250 mg/kg and 1000 mg/kg AIN-76A diet, respectively can suppress dimethylbenz(a)anthracene (60 mg DMBA/kg body weight at day 50)- induced mammary carcinogenesis in female Sprague Dawley rats. Next, we treated rats from birth throughout life with one of five treatments: 1) Control (AIN-76A diet only), 2) 333 mg resveratrol/kg diet, 3) 83 mg genistein/kg diet, 4) 333 mg resveratrol + 25 mg genistein/kg diet, or 5) 100 mg resveratrol + 83 mg genistein/kg diet (30/group). A significant reduction in mammary tumor multiplicity and a significant increase in tumor latency were observed in all four polyphenol treatments. Interestingly, adding a low dose (25 mg/kg diet) of genistein to the resveratrol treatment, we observed a further reduction in tumor multiplicity. As to the mechanisms of chemoprevention, analysis of mammary whole mounts revealed that genistein enhanced gland differentiation and reduced cell proliferation. Resveratrol significantly decreased epithelial cell proliferation and increased apoptosis in mammary terminal ductal structures, the most susceptible mammary structure to carcinogenesis. Microarray analysis at 50 days postpartum revealed 386 and 470 genes that were differentially regulated between the resveratrol- and genistein-treated rats (respectively) versus control rats at a p value ≤ 0.01. Many of these genes provide possible mechanisms to our findings on altered mammary gland differentiation, cell proliferation, apoptosis, and a reduction in carcinogenesis. Resveratrol treatment modulated many survival and apoptosis-related genes including Akt, several cyclin-dependant kinases, and Bad. Genistein in the diet affected several growth factor receptors including growth hormone receptor, IGF-2

receptor, and PPAR delta and other genes such as p53 and Bcl-2. The polyphenols genistein and resveratrol, alone and in combination, suppressed mammary tumorigenesis by modulating mammary gland differentiation and epithelial cell proliferation/apoptosis. More elucidation of these mechanisms should allow the use of these polyphenols in clinical trials and to prevent breast cancer. (Supported by NIH-NCI-P20-CA93753, DAMD17-00-1-0119, and DOD-BC043793)

3. AACR Annual Meeting. Proceedings, Volume 47, p. 534. Washington D.C. April 2006.

Mammary Cancer Chemoprevention with the Polyphenol Resveratrol

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Despite recent advances in therapeutic treatments, breast cancer remains a serious disease and a leading killer among female cancers. More than 200,000 new cases of breast cancer and 40,000 deaths in 2005 have been estimated. There is, and should be, a strong effort to work toward the prevention of this disease. It is well accepted that environmental factors, especially diet and lifestyle, play a critical role in determining one's risk for breast cancer. One dietary polyphenol that has received much attention for its health benefits, including anti-cancer properties, is resveratrol, a phytoalexin found in red grapes and red wine. We hypothesize resveratrol given in the diet can protect against chemically-induced mammary cancer through mechanisms that involve mammary gland differentiation and epithelial cell proliferation/apoptosis. Female Sprague Dawley rats were treated with 1000 mg resveratrol/kg AIN-76A diet from birth throughout life. At 50 days postpartum, animals were sacrificed to evaluate mammary whole mounts, cell proliferation, apoptosis, or treated with 60 mg dimethylbenz[a]anthracene (DMBA)/kg body weight to induce mammary adenocarcinomas. Tumor animals were palpated twice weekly for tumor appearance, location, and size. A follow-up tumor study with a lower dose of resveratrol (333 mg resveratrol/kg diet) was also done in the same manner. Gene expression arrays were done on mammary glands at 50 days postpartum for a further investigation of mechanisms of action. Both doses of resveratrol (1000 mg and 333 mg) were able to suppress mammary tumor multiplicity and increase tumor latency in a statistically significant manner. Mammary whole mount analysis revealed an increase in lobular structures, the least susceptible mammary terminal ductal structures to carcinogens. Cell proliferation and apoptosis assays revealed a drastic decrease in mammary epithelial cell proliferation and a significant increase in apoptosis in mammary terminal end buds, the most susceptible structures to carcinogenesis in the mammary gland. Microarray analysis at 50 days postpartum revealed 470 genes that were differentially regulated between the resveratrol-treated and control rats at a p value ≤ 0.01. Many of these genes confirmed or provide possible mechanisms to our findings on altered cell proliferation, apoptosis, and a reduction in carcinogenesis. Resveratrol treatment decreased several survival and cell cycle regulatory genes including Akt and several cyclin-dependent kinases. Also, resveratrol up-regulated the pro-apoptotic gene Bad and Metastasis Suppressor 1, while it

down-regulated CYP1A1 and CYP1A2. We conclude that resveratrol in the diet can protect against mammary carcinogenesis by modulating mammary gland differentiation, and epithelial cell proliferation and apoptosis. (Supported by NIH-NCI-P20-CA93753, DAMD17-00-1-0119, and DOD-BC043793)

4. Gordon Research Conference: Hormone Action in Development and Cancer. Holyoke, MA. July 2005.

Breast Cancer Chemoprevention with the Polyphenol Resveratrol.

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Even with the advent of new and aggressive therapeutics, breast cancer remains a destructive disease and a leading killer among cancers. In the U.S. alone, there are more than 215,000 new cases expected this year. There is, and should be, a concerted effort to prevent breast cancer. One polyphenol that has received much attention for health benefits is resveratrol, a polyphenolic phytoalexin found in red grapes (red wine). We have investigated the chemopreventive properties of resveratrol using the dimethylbenz(a)anthracene (DMBA) model for mammary carcinoma in the female Sprague-Dawley rat. Dietary administration of pure resveratrol (1g/kg AIN-76A diet) was provided, starting at parturition. Control animals were fed the phytoestrogen-free diet, AIN-76A. This dose of resveratrol resulted in a serum concentration of 2 ± 0.64 μM. With at least 30 animals per group, tumor multiplicity, and latency were observed. Rats exposed to resveratrol via the diet showed a decrease in tumor multiplicity and had a longer latency as compared to control animals. Resveratrol in the diet resulted in no significant effect on body and uterine weights in 21 and 50 day old rats. Analysis of mammary whole mounts showed that 21-day-old rats had fewer terminal end buds, the most susceptible structures for carcinogenesis, and at 50 days displayed more lobules, the most differentiated structures. Bromodeoxyuridine incorporation studies revealed that dietary resveratrol resulted in a significant reduction in the number of proliferative cells in the terminal ducts and terminal end buds. Towards a cellular mechanism of action we have examined another hormone responsive organ, the uterus. In 50-day-old female rats, treatment with dietary resveratrol resulted in reduced protein levels of the family of steroid receptor coactivators (SRCs). SRC-1, GRIP-1, and AIB1 protein levels were all reduced following resveratrol exposure. While these coactivators are reported to increase the transcriptional activity of steroid receptors including estrogen, progesterone, and androgen receptors, resveratrol may attenuate sex steroid action in multiple hormone-dependent organs by down-regulating their concentrations. The elucidation of the mechanisms for the preventive effects that we observe following polyphenol treatment will hopefully pave the way for clinical trials and the prevention of breast cancer. (Supported by NIH NCI P20 CA93753-02 and DOD DAMD17-00-1-0119).

5. Society of Toxicology 44 Annual Meeting. Program page 164. New Orleans, LA. March 2005.
Breast Cancer Chemoprevention with the Polyphenol Resveratrol.

Timothy Whitsett and Coral A. Lamartiniere. University of Alabama at Birmingham, Birmingham, AL 35205.

Even with the advent of new and aggressive therapeutics, breast cancer remains a destructive disease and a leading killer among cancers. In the US alone, there are more than 215,000 expected this year. There is and should be a concerted effort to prevent breast cancer. We have shown that the consumption of genistein, a major constituent of soy, in a prepubertal rat confers protection against chemically-induced mammary cancer. Another polyphenol that has received much attention for health benefits is resveratrol, a polyphenolic phytoalexin found in red grapes (red wine). We have investigated the chemopreventive properties of resveratrol using the DMBA model for mammary carcinoma in the Sprague-Dawley rat. Dietary administration of pure resveratrol (1g/kg AIN-76A diet) was provided for the entirety of life. Control animals were maintained on AIN-76A diet, reportedly void of phytoestrogens. This dose of resveratrol gave a serum concentration of 2 ± 0.64 μM. With at least 30 animals per treatment, tumor incidence, multiplicity, and latency were observed. Rats exposed to resveratrol via the diet showed a decrease in tumor multiplicity and had a longer latency as compared to control animals. As for mechanisms of action, resveratrol treated animals showed an increased number of mammary terminal end buds and lobules at 21 days of age, indicating increased cell proliferation and differentiation. In the uteri of animals treated with resveratrol, we saw a decrease in protein levels of the steroid receptor coactivators: SRC-1, GRIP-1, and AIB1. These coactivators increase the transcriptional activity of a host of steroid receptors including estrogen, progesterone, and androgen receptors, and others. We have shown that genistein treatment can regulate GRIP-1 protein levels in the rat mammary gland, with an increase at 21 days followed by a decrease at 50 days, a susceptible time to carcinogen exposure. The elucidation of the mechanisms for the preventive effects that we observe following polyphenol treatment will hopefully pave the way for clinical trials and the prevention of breast cancer.